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14. ABSTRACT

The mechanisms underlying the pathogenesis of dengue hemorrhagic fever (DHF) remain poorly understood, but a clear correlation appears to exist between dengue viral load and disease severity. While antibody-dependent enhancement and T-cell-mediated pathogenesis theories suggest an immunopathologic basis for the development of severe disease, intriguing evidence suggest a role for viral strain differences. Consistent genetic differences exist in the envelope glycoproteins of dengue 2 strains associated with DHF epidemics (Asian genotype) and dengue 2 strains only associated with DF (American genotype). It has also been established that dengue virus infection can be mediated by Civ type lectins DC-SIGN and L-SIGN. DC-SIGN is found on dendritic cells, a presumed target in human infection. L-SIGN is found on liver sinusoidal endothelial cells. Such cells have been shown to tolerize naïve T cells. To avoid the pitfalls associated with current measures of infection and neutralization, we developed an assay that uses cells expressing these relevant lectin receptors and low-passage viral isolates that yields results within 24 hours. Using this assay, we examined whether Asian and American genotype dengue 2 viruses exhibit differences in utilization of these two receptors. Our results showed that American strains infect DC-SIGN bearing cells to a greater extent than LSIGN bearing cells while Asian strains preferentially infect L-SIGN bearing cells. A single mutation in the envelope glycoprotein of an American strain at E390 from aspartic acid (American) to asparagine converted the C-type lectin binding phenotype from an American strain to an Asian strain by the observation that the E390 amino acid (aa) in the Asian strain is also asparagine. Furthermore, Asian and American strains differed in their sensitivity to antibody neutralization. The neutralizing capacity of mAbs 3H5 and 4G2 for Asian virus was significantly decreased when infection was measured in L-SIGN bearing cells compared to DC-SIGN bearing cells. We also found that serum from Venezuelan DF patients had much greater neutralizing capacity for Asian virus in LSIGN cells than serum from patients who progressed to DHF. Further, the magnitude of neutralization of L-SIGN-mediated Asian virus infection was inversely associated with disease severity. Taken together, our studies suggest that differences in receptor utilization and neutralization sensitivity between Asian and American dengue 2 strains may contribute to our understanding of the role that viral strain differences play in dengue pathogenesis.

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ABSTRACT

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type lectins DC-SIGN and L-SIGN. DC-SIGN is found on dendritic cells, a presumed target in human infection. L-SIGN is found on liver sinusoidal endothelial cells. Such cells have been shown to tolerize naïve T cells. To avoid the pitfalls associated with current measures of infection and neutralization, we developed an assay that uses cells expressing these relevant lectin receptors and low-passage viral isolates that yields results within 24 hours. Using this assay, we examined whether Asian and American genotype dengue 2 viruses exhibit differences in utilization of these two receptors. Our results showed that American strains infect DC-SIGN bearing cells to a greater extent than L-SIGN bearing cells while Asian strains preferentially infect L-SIGN bearing cells. A single mutation in the envelope glycoprotein of an American strain at E390 from aspartic acid (American) to asparagine converted the C-type lectin binding phenotype from an American strain to an Asian strain by the observation that the E390 amino acid (aa) in the Asian strain is also asparagine. Furthermore, Asian and American strains differed in their sensitivity to antibody neutralization. The neutralizing capacity of mAbs 3H5 and 4G2 for Asian virus was significantly decreased when infection was measured in L-SIGN bearing cells compared to DC-SIGN bearing cells. We also found that serum from Venezuelan DF patients had much greater neutralizing capacity for Asian virus in L-SIGN cells than serum from patients who progressed to DHF. Further, the magnitude of neutralization of L-SIGN-mediated Asian virus infection was inversely associated with disease severity. Taken together, our studies suggest that differences in receptor utilization and neutralization sensitivity between Asian and American dengue 2 strains may contribute to our understanding of the role that viral strain differences play in dengue pathogenesis.

TITLE PAGE

Differential Dengue Tropism & Neutralization: Potential Mechanisms of Pathogenesis

by

Nicole C. Martin

Thesis/dissertation submitted to the Faculty of the Emerging Infectious Diseases Graduate Program, Uniformed Services University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2006.

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DEDICATION

To my beloved husband, Steven

And my beautiful daughter, Brianna

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. CHAPTER 1: DENGUE

1.1. Theories of Pathogenesis

Dengue is currently considered the most important mosquito-borne viral disease affecting humans. With a global distribution comparable to malaria, an estimated 2.5 billion people live in areas at risk for epidemic transmission (Fig. 1-1). Tens of millions of cases of dengue fever (DF) occur each year and, depending upon the year, up to hundreds of thousands of dengue hemorrhagic fever (DHF) cases. In most countries, the case fatality rate of DHF is about 5%, with the most fatal cases occurring among children and young adults (282).

Belonging to the family *Flaviviridae*, dengue viruses consist of four antigenically distinct serotypes and are transmitted to humans by the bite of infected *Aedes* mosquitoes. The primary vectors of dengue virus transmission include *Aedes aegypti, A. albopictus, and A. polynesiensis* (228). Each serotype provides specific lifetime immunity and short-term heterologous immunity. Within each serotype there are genetic variations sufficient to generate different strains. Some strains appear to be more virulent or have more epidemic potential than others. Human infection may be asymptomatic or manifest as DF, characterized by fever, chills, frontal headache, myalgia, arthralgia, and rash. More severe manifestations of the disease, involving plasma leakage and hemorrhage (DHF) and shock (dengue shock syndrome-DSS) can also occur in a subset of individuals after resolution of acute fever. While the mechanisms underlying DHF pathogenesis remain

poorly understood, there is a clear correlation between dengue viral load and disease severity (269). A number of models have been proposed to explain the increased viremia, including antibody-dependent enhancement (ADE), T-cell cross-reactivity, and viral strain differences.

1.1.1. Antibody-Dependent Enhancement

The theory of ADE as an explanation for the pathophysiology of severe disease evolved out of the numerous epidemiological observations that a secondary infection with a heterotypic serotype markedly increases the risk for DHF and DSS (129, 243, 258). According to the ADE hypothesis, individuals with pre-existing antibody to one dengue virus serotype are predisposed to DHF when subsequently infected by a different dengue virus serotype. Pre-existing antibodies, under conditions of antibody specificity or concentration where neutralization does not occur, presumably mediate the "enhanced" infection. These antibodies complex with the virus and increase the efficiency of the Fc-receptor-bearing cells to take up the virus, thus resulting in increased infection. These cells can then induce elaboration of cytokines that modulate vascular permeability and subsequent symptoms seen in DHF/DSS.

In support of the ADE hypothesis, many prospective seroepidemiologic studies found that DHF epidemics consistently occurred when multiple types of dengue viruses were co-circulating and patients were experiencing secondary antibody responses (129, 243, 258). One study conducted in Thailand during 1980 found that previous monotypic dengue immunity was associated with increased risk for DHF, as eight of 36 children with secondary infections, but none of 27 with primary infections was hospitalized for DSS (243). Another study in Thailand found that sixteen percent of secondary infections

were symptomatic, compared to nine percent of primary infections and seven of 56 secondary infections, but none of 47 primary infections required hospitalization for DHF (129).

In addition to seroepidemiologic studies, experimental data have also provided support for the ADE hypothesis. In a prospective study of dengue in school children in Bangkok, undiluted pre-infection sera of children who had DHF were more likely to result in enhancement when tested *in vitro* than the pre-infection sera of children who had asymptomatic secondary dengue infections (130). Additionally, ADE could explain DHF that occurs in primary infections in infants. When sera from mothers of infants with DHF were studied, the anti-dengue antibody titer in the mothers' sera correlated with the age of occurrence of DHF and with the known serum half-life of immunoglobulin (129). A variety of other studies also demonstrate that sub-neutralizing concentrations of antibodies *in vitro* and in primates increased virus titers (97, 99, 100).

Studies have shown that dengue can infect and proliferate in macrophages and dendritic cells (162, 284). In peripheral blood mononuclear cells (PBMCs) obtained from patients with dengue virus infection, infectious virus was detected in the monocytes and tissue macrophages (247). Enhanced infection of these cells could result in more virus production than would otherwise occur. Several groups have studied the effects of dengue virus infection on monocyte function and resulting cytokine production. Infection of the monocytic cell line THP-1 with dengue virus induces TNF−α production (155). Additionally, culture supernatant from antibody-enhanced dengue virus infection of primary human monocytes activates endothelial cells in culture (4, 24), and this activation is blocked by adding antibody to TNF−α (4). Thus, dengue virus infection of

human monocytes may be enhanced in the presence of antibodies, leading to enhanced viremia and production of vasoactive factors.

While dengue virus infection of cells of the myeloid lineage, in the absence or presence of enhancing antibodies, has been extensively documented (26, 97, 101, 102, 130, 139, 175, 180, 214) few studies have used low-passage viral isolates (61, 172, 189). When low-passage viral isolates were used, productive infection was detected only in the human monocytic cell line, U937 cells (61). Even at the highest multiplicity of infection (MOI), and in the presence of enhancing antibodies, infection was undetectable by either flow-cytometry or viral plaque assays of THP-1 cells, PBMCs, and monocyte-derived macrophages (61, 172). Thus, the evidence regarding the ADE hypothesis is contradictory, leading some investigators to consider the possibility that ADE may be a laboratory anomaly.

1.1.2. T cell-Mediated Pathogenesis

In addition to the antibody-mediated arm of acquired or specific immunity, T lymphocytes constitute the cellular arm. T lymphocytes consist of two types: helper T cells ($CD4^+$) and killer T cells ($CD8^+$). Helper T cells direct the activity of killer T cells and are essential for an effective immune response since they activate other immune cells including most B cells to produce antibody. A complex network of cytokines is secreted by the helper T cells that determine the course of the immune response. Killer T cells, on the other hand, release cell-damaging enzymes that create holes in the membranes of the target cells and trigger them to undergo apoptosis. Individual T cells are targeted against the specific antigen signatures of viruses and bacteria, and when helper T cells encounter their specific antigens, they become activated and quickly expand in number.

It has been shown that marked T-cell activation and the levels of several T-cell cytokines, such as IFNγ and TNF−α correlate with disease severity (234, 290). Dengue virus-specific $CD4^+$ T lymphocytes have been shown to produce high levels of IFNγ (147, 150) IL-2, TNFα, and lymphotoxin (76). Dengue virus-specific $CD8^+$ T cells also produce IFNγ, albeit at lower levels than $CD4^+$ cells (234). These cytokines could mediate capillary leakage via multiple direct and indirect effects on the vascular endothelium. High levels of IL-2 can induce plasma leakage (10) and TNF−α induces both plasma leakage and shock in animal models (263). Further, IFNγ enhances TNF−α production by activated monocytes (211) and interacts with TNF−α to activate endothelial cells *in vitro* (32).

T cells and T-cell cytokines have the potential to cause damage in the host. Lymphocytic choriomeningitis virus (LCMV), for example, is a noncytopathic virus, but when an infection leads to a high antigen load, combined with a large cytotoxic T-lymphocyte (CTL) response, immune damage to infected tissues can ensue (1). It has also been established in several mouse models that immune-mediated activation-induced cell death of $CD4^+$ and $CD8^+$ T cells leads to cytokine release and subsequent immunopathology (1, 48). Animal models have shown that this immunopathology occurs when low affinity T cells continue to secrete cytokines in the presence of residual antigen (273). The activation of these low affinity T cells may be explained by a phenomenon called original antigenic sin (204).

Original antigenic sin is a concept that implies the host response to a secondary antigenic challenge is dominated by the proliferation of cross-reacting memory cells from a primary infection. Original antigenic sin may be an advantageous phenomenon in that

it may allow the rapid mobilization of a memory response, as memory cells have a much lower threshold for activation compared to naïve cells (270). However, there is a risk that the clones activated by original antigenic sin may be of lower affinity and, therefore, less effective at clearing infecting virus. In fact, these responses may be immunopathogenic (3, 273). Mouse models have shown marked pathology when T cells specific to previously encountered viruses respond to subsequent viral infections (281). Previous studies showed that reinfection of mice with certain LCMV strains activated the less-effective primary T-cell response, resulting in impaired viral clearance (128). These cross-reactive T cells induced by the previously encountered virus may prevail over new high-affinity T cells because of their numerical advantage and reduced threshold as memory T cells. This phenomenon is referred to as immunodomination and may occur when cross-reactive T-cell responses evoked from even relatively unrelated viruses compete with newly activated T cells for antigen on the antigen-presenting cells (286).

Serotype cross-reactive dengue virus-specific CTLs induced during a primary infection may play a role in the immunopathogenesis of DHF during a secondary dengue virus infection (151). Elevated levels of soluble CD8 in human serum were found in children with DHF compared to those with dengue fever, supporting an immunopathologic role for $CD8^+$ CTLs (146). Another study found that a substantial fraction of T cells activated during a second infection had poor affinity for the antigenic peptides of the second virus serotype. Instead, these T cells had higher affinity to other, presumably previously encountered, dengue virus serotypes and so did little to clear the virus while contributing to the immunopathology (204). Additionally, $CD8^+$ CTL responses from a primary dengue virus infection have been characterized in adults who were infected with live tissue culture-passaged dengue viruses (84, 147, 150, 176, 194).

Both serotype-specific and serotype cross-reactive CTLs were isolated and several different patterns of cross-reactivity were observed: 1.) some CTL clones recognized some dengue virus serotypes, 2.) other clones recognized all four dengue virus serotypes, and 3.) still other clones recognized closely related flaviviruses (145). Notably, not all flavivirus-cross-reactive CTL clones isolated from dengue virus-immunized humans or mice recognized all four dengue virus serotypes (287). This CTL cross-reactivity with other flaviviruses suggests that protective and immunopathologic effects might occur during sequential infections with flaviviruses other than dengue virus. In fact, several studies have found that the antibody response to candidate live dengue virus vaccines was enhanced in subjects previously immunized with a yellow fever virus vaccine (64, 246).

Thus, the high viral load that correlates with dengue disease severity could be attributed to the expansion of cross-reactive T cells that have relatively lower affinity for the infecting serotype, while the resulting T-cell cytokines could be mediating dengue pathogenesis. It may also be that the high antigenic load associated with a second dengue infection may preferentially drive high-affinity T cells into apoptosis via activation-induced cell death, which would further increase the frequency of lower-affinity cells (48). Regardless, the host could be left with a weak and ineffective group of T cells to clear the infection.

Although no animal model of DHF currently exists, murine dengue virus-specific CTL responses are argued to be qualitatively similar to human dengue virus-specific CTL responses (235). In studies with mice, the order in which sequential viral infections occur appeared to affect the patterns of dengue virus serotype cross-reactivity in short-term T-cell lines and clones. In mice immunized with dengue 2, the main CTL responses were dengue 2 or 4 cross-reactive, with a low level of CTL recognition of the dengue 1 or 3

sequence. In mice immunized with dengue 3, the CTL responses to this epitope were broadly serotype cross-reactive (252). Murine CTL responses to West Nile Virus (WNV) and Kunjin virus also demonstrated nonreciprocal CTL flavivirus cross-reactivity (106). Another study found that primary dengue virus infection induces a complex population of flavivirus cross-reactive NS3-specific CTL clones in mice and suggest that CTL responses are influenced by the viral serotype (252). Thus, the order in which sequential flavivirus infections occur may influence disease manifestations. The levels of serotype cross-reactive T-cell responses have varied substantially in studies of dengue virus immunized humans as well, but these studies did not clarify whether the different CTL specificities were related to the viral serotype.

Epidemiological studies have also suggested that the order of acquisition of dengue virus infections is important. Specifically, having a sequence of infections in which dengue 2 is the agent of secondary dengue virus infection increases the odds of DHF or dengue shock syndrome (243, 258). Whether this phenomenon is due to the presence of antibody (ADE theory), and/or because the CTLs induced by other serotypes recognize dengue 2 virus to a greater extent than the reverse (T-cell theory) remains to be determined (252). Alternatively, it has been proposed that differences in virulence between the different dengue virus serotypes, or strains within those serotypes, explain this finding (45, 46, 59, 226).

1.1.3. Viral Factor

The ADE and cross-reactive T cell hypotheses do not account for observations that DHF does not always occur in areas where multiple serotypes of dengue virus co-circulate or when a new serotype is introduced into an area with pre-existing antibody

prevalence to a different serotype. During the 1970s and 1980s in the American tropics, Polynesia, and on the Indian subcontinent, three or four types of dengue viruses circulated sequentially and sometimes simultaneously, but without generating epidemic DHF/DSS (35, 87). Dengue 1 and dengue 2 both circulated in Latin America for several years without any cases of DHF (87). It was not until 1981 that cases of DHF emerged in the Americas; reports of DHF began with an explosive outbreak in Cuba and quickly spread throughout the rest of Central and South America (Fig. 1-2) (87, 93, 141). Genetic analysis revealed that the strain of dengue 2 associated with the DHF epidemics belonged to a different genotype (Asian) than the indigenous dengue 2 (American) that had previously been co-circulating with dengue 1 in Latin America (45, 46, 59, 159, 226, 227).

A prospective serosurvey was initiated in 1993-94 during a dengue 1 epidemic in Iquitos, Peru. Approximately four years later, and in the context of a high seroprevalence of anti-dengue 1 antibody, an epidemic of dengue 2 infections ensued. Despite a thorough investigation, no cases of DHF could be detected (277). It was later found that the strain of dengue 2 circulating during the second epidemic was the American genotype. In contrast, Venezuela experienced a significant burden of DHF disease with circulation of the Asian (Asian) genotype dengue 2 in a dengue 1 immune population. Asian or closely related dengue 2 viruses have subsequently caused large outbreaks of DHF in Venezuela and Brazil and smaller outbreaks in a number of other Central and South American countries, all areas where previous transmission of dengue 1 and American genotype dengue 2 occurred with no cases of DHF (87).

These observations illustrate the important role viral factors may play in dengue pathogenesis and demonstrate that, within serotype 2 viruses, a specific genotype may be

able to cause DHF, while other genotypes are associated with less severe disease. Thus, specific structural differences between the Asian and American genotypes may affect pathogenesis, and a viral factor may very well play a role in the occurrence of DHF/DSS, as this syndrome occurs at epidemic proportions only when the second infecting serotype is of Asian origin (45, 46, 59, 226).

The complete genomes of the American and Asian dengue 2 viruses have been sequenced and speculations made as to the potential structures involved in human virulence (158). When Asian genotype viruses were compared to American genotype viruses (227, 277), a total of 55 amino acid changes were consistently detected between the two genotypes, with 4 amino acid differences found in the envelope (E) glycoprotein. The virulence of several flaviviruses has often been associated with amino acid differences located the E glycoprotein (36, 177, 182, 219). One of the amino acid charge differences considered to be of major importance between Asian and American genotypes exists in the E glycoprotein at amino acid 390 (E390) (158). Whereas Asian genotypes have an asparagine (Asn or N) at amino acid 390 (E390), American genotypes have an aspartic acid (Asp or D). Additionally, envelope amino acid differences exist at 81, 139, and 162 (Table 1-1) (137). Whereas Asian genotypes have an isoleucine (Ile) at E139 and 162, American genotypes have a valine (Val) in both positions.

1.2. Dengue Envelope Glycoprotein

The envelope (E) glycoprotein of flaviviruses plays a significant role in viral entry and possesses an interesting structural and functional biology. Exposed on the surface of the virus, it mediates attachment of the virus to host cells, as well as fusion of the viral and cellular membranes after receptor-mediated endocytosis (Fig. 1-3) (42, 230). In addition, it is the major target of neutralizing antibodies in the host and plays an essential role in both viral tropism and pathogenesis. The three-dimensional structure of the ectodomain of the E protein has been determined for dengue (143) and tick-borne encephalitis virus (TBE) (224), which serves as a useful model for other flaviviruses, given the high amino acid sequence homology observed throughout the genus. The protein forms head-to-tail dimers on the virion surface and each monomer consists of three distinct functional domains named I, II, and III, which correlate well with the described dengue virus E antigenic domains C, A, and B (230).

Domain I is the central domain, II includes the dimerization region, and III contains the binding domain (Fig. 1-4). The molecular determinants of virulence on the flavivirus E protein are hypothesized to affect virulence by disrupting the functional biology of the protein (224). There is a putative hinge region that links domains I and II and contains a highly conserved "fusion peptide" (230). This hinge region plays an important role in early events during infection of the cell as it undergoes a pH-dependent conformational change that converts the E protein from a dimeric to a trimeric form and projects domain II outward, presumably bringing the tip into juxtaposition with the endosomal membrane (Fig. 1-5). The result is fusion of the viral envelope with the

endosomal membrane and subsequent virus release into the cytoplasm. Thus, mutations in the hinge region may alter the ability of the E protein to undergo this required conformational alteration at acidic pH, and therefore, disrupt normal fusion activity. Due to the dipolar nature of amino acids and significant differences in pK values, amino acid substitutions may result in marked differences in charge and hydrophilicity.

1.2.1. Mutations and Cell Tropism

Domain II of the envelope glycoprotein contains the hinge region and highly conserved fusion peptide. This region undergoes a pH-dependent conformational change that ultimately results in endosomal fusion. Studies with flaviviruses have demonstrated that mutations in this hinge region cause significant attenuation and affect neuroinvasiveness and neurovirulence by affecting the pH-dependent conformational change required for fusion (7, 36, 38, 39, 104, 244, 255). A single mutation in the hinge region of flavivirus JE envelope altered both neurovirulence and viscerotropism (203). A glutamic acid (Glu) 126->Lys amino acid substitution resulted in the acquisition of mouse neurovirulence of dengue 2 New Guinea C (28),(85). Guirakhoo *et al.* found a mutant of dengue 2 virus that contained three amino acid substitutions: Ile6->Met (both nonpolar hydrophobic), Asn134->Ser (both polar hydrophilic), and Asn153->tyrosine (Tyr) (both polar hydrophilic). Despite the similarity in both charge and polarity, these mutations still resulted in slower growth and smaller plaques in Vero cells and elevated pH thresholds of fusion, as compared to the parent virus (91). Asian and American strains of dengue 2 have different amino acids in two locations in the hinge region. The Asian strains have an Ile at both aa139 and aa162, while American strains has a Val in both positions (137). Even though these differences do not result in a change in charge or

polarity, previous studies indicate that growth rates and fusion requirements differ as a result of variations of certain key amino acids (7, 36, 38, 39, 104, 244, 255).

Domain III is known to play an important role in viral binding (158) with the lateral surface containing residues implicated as determinants for virulence, host range, and tropism in different flaviviruses (224). Thus, these mutations are predicted to disrupt receptor binding (224, 230) and have been shown to affect virus infectivity and virulence in flaviviruses (27, 28, 156, 177, 265). One of the observed differences in envelope glycoprotein between Asian and American dengue 2 genotypes resides in aa residue E390. Given that residue 390 is located in the putative receptor-binding domain (41, 224), a possible functional consequence of substitutions at this position is an altered receptor interaction.

Analysis of E390 mutants confirms the significance of this residue as a determinant of host cell tropism. Lee *et al.* constructed an infectious cDNA clone of flavivirus MVE and employed it to systematically investigate the impact of single amino acid changes at E390 on cell tropism, virus entry, and virulence (156). Mutations at E390 from the prototype Asp to Gly, Ala, and His resulted in pronounced differences in growth in mammalian and mosquito cells. The mutation to Gly or Ala resulted in a greater than 10-fold improvement of growth in a human cell line, but a concomitant 50 to 100-fold reduction of growth in Vero and C6/36 (*Aedes albopictus*) cells. The altered cell tropism correlated with a difference in entry kinetics, increased dependence on glycosaminoglycans, and the loss of virulence in mice (156). In addition, Sanchez and Ruiz have shown that a single nucleotide change in dengue 2 Mexican strain E390 affects neurovirulence in mice. A substitution of acidic Asp-> basic His at residue 390 resulted in increased virulence in suckling mice when inoculated intracerebrally, thus

demonstrating the important role E390 plays in viral binding and the substantial effect it has on virulence and cellular tropism (242).

The observed amino acid change at position E390 is also localized within one of the putative glycosaminoglycan binding motifs at amino acids 284 to 310 and 386 to 411 (42). These motifs have been shown to specifically bind dengue 2 virus to the host cell surface and blockage of this receptor with an antagonist prevented infection (42). Since glycosaminoglycan binding motifs in proteins are predicted by defining multiple regions enriched for basic amino acids (72), a change from acidic Asp (American) to neutral Asn (Asian) may affect dengue virus attachment to cells. In support of this, an uptake assay in which noneclipsed flavivirus MVE virus particles were inactivated by acid treatment, clearly demonstrated the faster entry kinetics of a more basic His390 mutant relative to the control virus (156).

The effect on viral tropism of single amino acid changes in the E glycoprotein is well documented for a number of flaviviruses (177, 264), including dengue (85, 221). The possibility that the E390 differences between Asian and American dengue 2 viruses can alter viral tropism may explain the correlation between viremia and pathogenesis. Prospective studies have shown that progression to DHF is associated with higher mean plasma viremias (161, 163, 269). The higher mean viremias observed for patients who develop DHF could be explained by more cells being infected in DHF patients, thus generating more virus. ADE has been proposed as a mechanism for the increased risk of developing DHF associated with secondary dengue virus infection and may explain, in part, the increased viremias associated with DHF. However, for dengue 2 American genotype viruses, secondary infection is not associated with an increased risk of developing DHF (277). Additionally, the likely initial targets for dengue virus replication

are immature dendritic cells in the skin and infection efficiency is not influenced by the presence of anti-dengue virus antibodies, indicating that immature DCs are not susceptible to ADE (284).

It would be advantageous to directly compare viral infection rates of Asian and American dengue 2 viruses in relevant cells. However, one of the major difficulties in directly comparing viral strains is achieving equivalent viral input inoculum. Typically, researchers use the same multiplicity of infection (MOI) or number of plaque forming units (pfu) per number of cells. However, this MOI is calculated from the virus titer, as determined by plaque assay in baby hamster kidney (BHK) cells or Vero cells. The assumption that the number of infecting units for BHK cells would be the same for dendritic cells may not be correct since virus entry and replication may be different for different cell types. American dengue 2 virus may have lower titers in BHK cells than Asian strains; however, American dengue 2 virus may infect dendritic cells more efficiently than the Asian strains. Equalizing the two genotypes based on their ability to infect BHK cells would mask this effect. Support in the field is growing to use an alternative to equalizing input inoculum based on virus titer. Researchers are now turning to equivalence based on genome copy number.

1.2.2. Viral Passage Adaptations

Flaviviruses undergo selective mutational pressures during replication in different host systems. Not surprisingly, the virus adapts to and becomes more fit for the cells in which it is being passed, while fitness declines for other cell types. Eastern equine encephalitis (278) and dengue viruses (40, 278) adapted to mammalian cells replicate more poorly than the parent in mosquito cells, while mosquito-adapted virus exhibits a

fitness decline in mammalian cells. Exploiting this phenomenon, the generation of attenuated virus for candidate dengue vaccines was initially achieved by serial intracranial passage in mice which resulted in an increased neurovirulence for mice, but a progressive loss in pathogenicity for humans (108, 238). Today, most candidate dengue vaccines have been generated by passage in cell culture.

Sequence changes accompanying cell culture passage point to the envelope (E) protein as the main site of mutations (177) and reveals the important role it plays in attenuation of YF (94), JE (213), and Murray Valley encephalitis (195). Hahn *et al.* compared the nucleotide and deduced amino acid sequences of the virulent Asibi strain of YF with that of the 17D vaccine strain derived from it by 240 passages in chick tissues (94). They found that 12 of 32 amino acid substitutions in the encoded proteins were in E. Likewise with MVE, after 10 passages in monkey kidney cells (Vero), the small number of amino acid substitutions which took place was also focused on the E protein and resulted in a decrease in mouse neuroinvasiveness and decreased yields in C6/36 cells (195). Similarly, adaptation of TBE virus by serial passage in BHK-21 cells resulted in mutations in the E protein that increased binding affinity for BHK-21 cells, but reduced neuroinvasiveness of the virus in adult mice (183). In JE as well, mutations in the envelope protein reduced virulence in mice and ablated entry into cultured Vero cells (104).

It has been empirically observed that adaptation by serial passage of dengue in mouse brain results in enhanced neurovirulence, but attenuation of viscerotropism (28). Substitutions in DEN-1 virus E protein during mouse brain tissue-specific adaptation may change the affinity of binding to receptors on target cells or affect the entry of virions by altering the fusion-regulating structural change within the virus particle (157, 196). It has

been reported that certain mouse-adapted variants of DEN-1 virus replicate less efficiently than the parental virus in non-human primate cells (28). Further, neuroadaptation of dengue 1 virus resulted in mutations in E that were associated with attenuation in human hepatoma cells, a presumed marker of viscerotropism (63).

Unrecognized adaptive mutations occurring during propagation of flaviviruses in the laboratory can be a source of misleading results and erroneous conclusions regarding the viral life cycle in the natural host (14). It has been demonstrated that adaptation to certain cell lines results in the selection of mutants that bind heparan sulfate with high affinity and display high levels of infectivity *in vitro*, but are attenuated *in vivo* (15, 33, 133). In contrast, the virulent wild-type strains do not bind efficiently to HS and exhibit much lower infectivity for cultured cells than HS-binding laboratory strains (15, 131, 133). Mandl *et al.* reports the emergence of multiple potential HS binding sites distributed over the surface of the E protein of TBE virus as a result of adaptation to growth in BHK cells (183).

To help define the molecular events involved in dengue virus adaptation during serial passage *in vitro* and in cultured cells, Lee *et al.* sequenced the structural protein genes of three dengue 3 isolates after intracerebral passage in mice and after passage in cultured monkey kidney (Vero) and *Aedes albopictus* (mosquito) cells (157). Two independent passage series in mosquito cells each resulted in the loss of a conserved glycosylation site at Asn153 in E, consequently altering fusion characteristics (157). Numerous other studies have shown that passaging dengue virus results in altered glycosylation which affects both virulence and cell tropism (91, 126, 221).

1.2.3. Effects of Differential Glycosylation

Glycosylation is common form of post-translational modification of proteins which occurs as two basic types, oligosaccharide side chains are either N-linked to asparagine residues or O-linked to hydroxyl groups of serine and threonine residues. Most viral glycoproteins are structural proteins that become incorporated into the membrane bilayer of enveloped viruses. Glycosylation of these envelope proteins plays an essential role in the establishment of their bioactive conformation and also has effects on receptor binding, fusion activity, and antigenic properties of viruses (2, 25, 245).

There are two potential glycosylation sites on the dengue E glycoprotein: Asn67 and Asn153 (Fig. 1-6). Not all dengue serotypes are glycosylated on both sites, although Asn67 is presumably conserved among many strains (226, 227). The second glycosylation site in E maps to the central domain I and the removal of this site and its corresponding N-linked glycan may affect the rearrangement of E and fusion activity (157). Dengue 4 neurovirulence has been shown to be significantly affected by the loss of this second glycosylation site. A single substitution of Ile for Thr155 ablated the second glycosylation site, and resulted in a virus that was almost as neurovirulent as a mouse-adapted mutant (126). These results suggest that glycosylation sites play an important role in virulence potential.

Rey *et al.* suggested that both the hinge region of the TBE E protein and the N-linked oligosaccharide at E154 may be involved in flavivirus fusion with cell membranes, as the sugar at E154 lies over a hydrophobic groove and most likely participates in stabilizing the E dimer (224). Consistent with those speculations, Guirakhoo *et al.* found that mosquito cell-passaged dengue 2 variants lacking the corresponding glycosylation site show an elevated fusion pH threshold compared with parental virus, which suggests

that fusion of the viral envelope with cellular membranes may be a point at which selective pressures can be exerted during a limited number of passaging steps (91).

In summary, laboratory adaptations are common and occur rapidly. It is likely that laboratory stock viral strains have suffered various mutations via years of serial passaging, and therefore could differ significantly from the virus populations circulating in nature. These findings underscore the potential importance of using low passage viral isolates in neutralization assays to reduce mutations and subsequent altered cell receptor usage from potential changes in glycosylation sites.

1.3. Candidate Dengue Virus Receptor

Despite the considerable homologies among flaviviruses, these viruses show a remarkable capacity to cause vastly different diseases with a minimum of alterations in the E protein. Regardless of whether genetic differences exist *de novo* among strains, or whether mutations arise from serial passaging or changes in glycosylation sites, small differences can have significant impact on virulence and cellular tropism studies. The ability of dengue virus to cause divergent clinical syndromes as well as the virus's association with replication in several organs have profound implications for the types of cell surface molecules that the virus recognizes as receptors. Small changes in viral glycoproteins may dictate which receptors can be used and influence the types of cells that can be infected (109, 110, 164). A precedent for a change in cellular tropism during an ongoing infection exists, since many HIV-1 infections initially involve macrophage tropic strains that, through minor changes in the envelope glycoprotein, shift to become

T-cell tropic and subsequently use an alternate receptor for entry (13, 49). Minor changes in the viral attachment proteins of other viruses have also been associated with changes in receptor usage.

A wide variation in the abilities of different dengue virus 2 isolates to productively infect cells *in vitro* has been observed, as judged by the accumulation of viral RNA and antigen and the generation of infectious virus in cell supernatants. Despite the use of equivalent multiplicities of infection, infection depended on the virus genotype and passage history (61). In addition, dengue virus 2 strains isolated from patients with various disease severities produced different levels of infectious virus in LLC-MK2 cells (184), peripheral blood leukocytes, and C6/36 cells (208).

Due to *de novo* genetic differences and the striking ability of flaviviruses to adapt to different cells and receptors during serial passaging, it is not surprising that the identification of dengue virus receptor(s) on target cells is still not definitive. Early studies described a cell-surface protein on human monocytes responsible for binding of dengue virus in the absence of virus-specific antibodies (i.e. a non-FcR molecule) (57). In contrast, other investigators using a recombinant dengue virus envelope-Fc fusion protein were unable to detect binding to human monocytes other than via the FcR (42). A series of reports describing dengue virus-binding molecules on both human, non-human mammalian and insect cell lines have implicated glycoproteins (188, 241), glycosaminoglycans (GAGs) (41, 114), and a lipopolysaccharide (LPS) binding $CD14^-$ associated molecule (43) in the dengue virus attachment process. Taken together, these studies and others have demonstrated that the dengue virus-binding moieties on the cell-surface membrane may vary between cell types and species origin, as well as among dengue virus serotypes (18, 19). Moreover, most of these studies have used cell types

other than those thought to be the main target cells in humans, which are dendritic cells (284). Recently, the C-type lectins DC-SIGN and L-SIGN have been shown to mediate dengue virus infection *in vitro* (257)

1.3.1. C-type Lectins

C-type lectins are proteins that bind carbohydrates in a calcium-dependent manner using highly conserved carbohydrate recognition domains (CRDs). The C-type CRDs are incorporated in a variety of contexts of molecular organization and are categorized into either soluble C-type lectins secreted from cells or transmembrane C-type lectins. The latter form consists of Type I and Type II lectins based on the number of CRDs present, but both receptor types contain the prototypic lectin fold, consisting of two anti-parallel β strands and two α helices (Fig. 1-7) (138). C-type lectins can be found on numerous cell types in a wide variety of tissues and can mediate cell-adhesion, regulation of signaling pathways, and recognition of specific carbohydrate structures present on self antigens and pathogens (70). Despite the similarities of C-type lectins and possible redundancy, the specificity for ligands can differ greatly. The complexity of the mannose structures recognized, the number of mannose groups and their branching and spacing on the ligand, as well as additional interactions other than carbohydrates, contribute to ligand specificity.

Many different C-type lectins have been described, including the macrophage mannose receptor (MMR/CD206), DEC 205 (CD205), DC-specific ICAM-3 grabbing non-integrin (DC-SIGN/CD209), blood DC antigen 2 (BDCA2), dectin-1, DC immunoreceptor (DCIR), DC-associated lectin 1 (DCAL1), C-type lectin receptor 1 (CLEC1), Langerhans-cell-specific C-type lectin (Langerin, CD207), DC-

asialoglycoprotein receptor (DC-ASGPR)/macrophage galactose N-acetyl-galactosamine specific lectin 1 (MGL1), and more recently DC-SIGN-related (DC-SIGNR/L-SIGN/CD209L) lectin (70). C-type lectin expression depends on cells' tissue localization, differentiation, and state of activation. Blood DCs, Langerhan cells (LCs) and *in vitro* cultured immature monocyte-derived DCs express only low levels of DEC-205, but expression greatly increases upon activation (70). In contrast, immature monocyte-derived DCs express abundant MMR and DC-SIGN, but production diminishes upon maturation (123, 257). *In situ* experiments have located DC-SIGN-expressing cells in the T-cell area of lymph nodes, tonsils, and spleen, as well as in the mucosal tissues such as rectum, cervix and placenta, and in skin sections DC-SIGN is expressed by dermal DCs (78). The DC-SIGN homologue designated DC-SIGNR (250) or L-SIGN (Liver/lymph node-specific ICAM-3 grabbing non-integrin) (12) shares 77% amino acid sequence identity with DC-SIGN (250) and is expressed by endothelial cells in lymph nodes, gastrointestinal tract, placenta, and by liver sinusoidal endothelial cells (LSECs) (68).

Most C-type lectins expressed by DCs are type II transmembrane proteins with the exception of the mannose receptor and DEC205, which are both type I transmembrane proteins (70). All type II C-type lectins contain one extracellular CRD (280) whereas the mannose receptor and DEC205 contain eight and ten CRDs, respectively. In addition to the extracellular CRD(s), two Ca^{+2} binding sites are present on a loop protruding from the protein surface. Mutation of these sites results in a loss of ligand binding, demonstrating the dependence on calcium (79). The CRD of DC-SIGN is separated from the transmembrane region by a neck domain that consists of seven complete and one incomplete repetitive sequences. These sequences are thought to affect

the formation of oligomers and subsequently influence carbohydrate specificity (199). Oligomerization of lectin domains has been shown previously to alter the affinity and specificity of carbohydrate recognition (279). Whereas the mannose receptor and MGL1 form trimers, DC-SIGN has been shown to form tetramers (199) which property could contribute to distinct carbohydrate-binding specificities (68).

The presence of a Glu-Pro-Asn site in the CRD of DC-SIGN, mannose receptor, Langerin, and BDCA2 predicts specificity for mannose-containing carbohydrate structures. However, each recognizes distinct mannose structures due to different branching and spacing (78, 268). Whereas the mannose receptor recognizes end-standing single mannose-branched structures or di-mannose clusters, DC-SIGN recognizes both internal mannose branched structures with a minimum of three mannoses (high mannose) and end-standing di-mannoses (68, 78). The valine residue in the CRD of DC-SIGN has been shown to be important for the recognition of only some ligands, suggesting different, but overlapping, binding sites. Furthermore, the recognition of specific carbohydrate structures by DC-SIGN seems to depend on the spacing of the carbohydrate structures on a glycoprotein. Screening panels of synthetic glycoconjugates that contain mannose, galactose or fucose residues and their multimeric derivatives have further determined the specificity of C-type lectins. Results indicated that DC-SIGN has a high specificity for fucose-containing carbohydrates, such as Le^x and for complex mannose-containing glycoconjugates (6, 68, 199).

DC-SIGN recognizes the self glycoproteins intercellular adhesion molecule 2 (ICAM2) and ICAM3, and functions as a cell-adhesion receptor that regulates DC migration (267) and DC-T-cell interactions (78), respectively. By binding to endothelial ICAM2, DC-SIGN acts as a tethering and rolling receptor on DCs to mediate chemokine-

induced transendothelial migration from blood to tissues (79). In this way, DC-SIGN functions similarly to selectins, which are also members of the C-type lectin family, and mediate leukocyte rolling and migration (78). The interaction of DC-SIGN with ICAM-3 stabilizes the intimate DC-T-cell membrane contact and allows T cells to screen the peptide-MHC complexes and enables efficient engagement of the T-cell receptor (TCR) through which signaling can occur and subsequent T-cell activation. The importance of DC-SIGN-ICAM3 interactions in the initial DC-T-cell contact is emphasized by the potency of DC-SIGN-specific antibodies to inhibit DC-T-cell clustering and DC-induced proliferation of resting T cells (78). Both ICAM2 and ICAM3 are heavily glycosylated glycoproteins that potentially contain high mannose-type oligosaccharides (75). Enzymatic removal of the N-linked carbohydrates from ICAM2 and ICAM3 abrogates binding to DC-SIGN (79). Glycosylation of ICAM2 and ICAM3 are cell-specific and mediated by the expression of different glycosyltransferases and glycosidases, which add or remove certain carbohydrate residues. Because carbohydrates interact specifically with lectins, altered glycosylation of a glycoprotein can modify its recognition by C-type lectins (55). Thus, given that dengue infection can be mediated by C-type lectins, *de novo* genetic differences between viruses or mutations in viral envelope glycoproteins could significantly impact viral binding and therefore cell tropism.

Another function of DC-SIGN is to interact with conserved molecular structures present on a wide variety of microorganisms and internalize these pathogens for processing and subsequent antigen presentation (62, 123). Depending on tissue localization and differentiation state, DCs are specialized to respond to specific microorganisms by expressing distinct sets of C-type lectins. Several membrane-bound C-type lectins on DCs function as endocytic receptors and are either constitutively

internalized from the cell surface like MMR (253), or are internalized upon ligand binding like DC-SIGN and DEC205. The cytoplasmic tail of DC-SIGN contains internalization motifs, such as the di-leucine (Leu-Leu) motif, tri-acidic (Glu-Glu-Glu) cluster and an incomplete immunoreceptor tyrosine-based activation motif (ITAM) (65). The di-leucine motif appears to be essential for rapid internalization of soluble ligands, while the tri-acidic cluster enables internalized antigens to be targeted to lysosomal compartments where ligands are processed for MHC class II presentation to T cells (268). In contrast, other C-type lectins, such as the mannose receptor, quickly recycle through early endosomes, resulting in large amounts of antigen uptake.

Given that DC-SIGN binds distinct carbohydrate structures, such as mannose-containing glycoconjugates and fucose-containing Lewis blood-group antigens, indicates that the carbohydrate specificity of DC-SIGN governs a broad range of pathogen-recognition. In fact, DC-SIGN functions as an attachment receptor for several bacteria (*Helicobacter pylori, Mycobacteria tuberculosis* (6, 256)), parasites (*Leishmania pifanoi, Schistosoma mansoni* (47, 266)), yeast (*Candida albicans* (34)) and many viruses, including both macrophage and T-cell tropic HIV-1, HIV-2 and simian immunodeficiency virus (SIV), Ebola virus, cytomegalovirus (CMV), hepatitis C virus and dengue virus (95, 210, 220, 248, 257). DC-SIGN recognizes the relatively large number of N-linked carbohydrates contained on the viral E glycoproteins expressed by these different viruses.

However, a degree of specificity in C-type lectin binding does exist. Viruses that express heavily glycosylated glycoproteins on their cell surface, such as vesicular stomatitis virus, fail to interact with DC-SIGN (153, 248). Furthermore, murine leukemia virus, which typically does not interact efficiently with DC-SIGN, could do so when

produced in the presence of a mannosidase I inhibitor (170). In fact, it has been shown that modulation of the content of immature carbohydrate chains on a glycoprotein can affect lectin binding (68, 170, 199). For HIV-1, Ebola, and dengue virus, it has been demonstrated that differential glycosylation of the E glycoprotein affects binding of DC-SIGN and the capacity to enhance infection of target cells (132, 169, 178). This differential glycosylation of a viral glycoprotein depends on the cell type producing the virus or is due to genetic *de novo* viral differences.

1.3.2. Binding Specificities of SIGN Lectins

Differential glycosylation occurs in virus derived from mosquito versus mammalian cells and results in differential binding to DC-SIGN and L-SIGN. Sindbis virus produced in mosquito cells or under conditions that limited the processing of viral carbohydrate modifications exhibited increased binding and infectivity for DC-SIGN- and L-SIGN-expressing cells as compared to mammalian cell-produced virus (132). Smith *et al.* demonstrated the decreased glycosylation of virus produced in mosquito cells when he showed that the E glycoprotein from dengue 2 virus grown in C6/36 cells migrated faster through polyacrylamide gels containing SDS than E from virus grown in Vero cells (249). Thus, the presence of immature, mannose-containing carbohydrate structures, not fully processed during transport through the Golgi apparatus, appears to be required for efficient binding to SIGN lectins, perhaps explaining why some glycosylated viruses do not bind DC-SIGN (248).

In addition to the divergence in carbohydrate processing between insects and mammals and its differential effects on DC-SIGN and L-SIGN binding, viral glycoprotein carbohydrate modifications may also differ between closely related species

and even within individual tissues of the same species. The differential glycosylation of viral envelopes due to differences in producer cell type and virus strain can impact interactions with DC/L-SIGN and possibly other C-type lectins. HIV glycoprotein carbohydrate modifications can exhibit diverse types of carbohydrate processing, depending upon the human host cell type that produced it. This property has been correlated with altered C-type lectin interactions (170). Envelope proteins from HIV produced in macrophages contains N-linked carbohydrate structures that are more complex than those found on virus produced in PBMCs (165, 166, 283). This differential glycosylation of HIV envelope affected interactions with DC-SIGN and L-SIGN in that HIV derived from PBMCs was bound and transmitted well by these lectins whereas HIV derived from macrophages was bound and transmitted poorly (170). Thus, virus transmissibility and tropism for cell types expressing specific lectins could vary during different phases of replication within a single host or between closely related hosts.

Differences in glycosylation of viral envelopes depend not only on the producer cell type, but on the viral isolate as well. Recent studies have shown that infection by Ebola virus Zaire glycoprotein pseudovirions could be enhanced by DC-SIGN and L-SIGN to a greater degree than Ebola virus Sudan (170). It was determined that this effect correlated with the fact that Zaire contained more high-mannose N-glycans than Sudan (69, 170). It is certainly plausible then that dengue virus glycoprotein N-glycan composition could vary between isolates, as well as within the same isolate depending on the cell type in which the virus is produced, and these differences could impact lectin-binding specificity such that either L-SIGN or DC-SIGN could be preferentially targeted.

1.3.3. L-SIGN

Despite the fact that DC-SIGN and L-SIGN share 84% amino acid sequence identity (Fig. 1-8) (250), evidence suggests that L-SIGN may have additional or slightly different requirements than DC-SIGN for efficient glycoprotein binding. Binding and infectivity of mosquito-derived Sindbis virus for cells bearing L-SIGN were lower than those for DC-SIGN and infectivity for the L-SIGN-expressing cells was further reduced four-fold when infection was conducted at 4°C, while DC-SIGN-mediated infection remained unaffected (132). The investigators propose that the interaction of mosquito cell-derived Sindbis virus carbohydrates with DC-SIGN is likely of higher affinity than that of L-SIGN and their data suggest that virus produced in and delivered by arthropod vectors would, due to enhanced high-mannose and $Man_3GlcNAc_2$ content, bind more efficiently to DC-SIGN and secondarily to L-SIGN and therefore preferentially targeting cells bearing these molecules (132). Similarly, using soluble hepatitis C virus E2 glycoproteins, monoclonal antibody 6/1a detected a version of the E2 glycoprotein bound to DC-SIGN, but not to L-SIGN, suggesting subtle differences in the interaction of these lectins with that virus (220).

Conversely, Venezuelan equine encephalitis virus replicon particles produced in mammalian BHK cells exhibited low infectivity for DC-SIGN-expressing cells, but were nevertheless infectious for L-SIGN-expressing cells (132). Likewise, differences in Ebola virus glycoprotein N-glycan composition affected the efficiency of DC-SIGN and L-SIGN enhancement of infection. Ebola virus glycoprotein pseudovirions produced in macrophages interacted with DC-SIGN poorly, but infection in L-SIGN expressing cells was enhanced (170). The authors suggest that the Ebola virion modulates its N-glycan status in order to emerge from the macrophage more efficiently (an important early target

of infection) by avoiding reattachment by DC-SIGN and allowing subsequent infection of other cell types, such as endothelial cells (170).

Increased serum levels of liver enzymes have been shown to correlate with the development of DHF (121). It has also been demonstrated that dengue virus can infect diverse liver cells with differing replication efficiency and result in cytopathic effects of varied severity (173). Examination of tissue from naturally-infected humans detected dengue antigen in cells of the reticuloendothelial system (71), which includes LSECs. LSECs are microvascular endothelial cells that express L-SIGN, interact with leukocytes via adhesion molecules, and constitute a central mechanism of peripheral immune surveillance in the liver (Fig. 1-9) (134). Another study recently demonstrated the presence of dengue viral antigen specifically in the LSECs (118), which is not surprising given that they are strategically positioned in the hepatic sinusoids to come into contact with antigens in the blood. LSECs are also perfectly positioned to come into contact with T cells, and they share a variety of features with dendritic cells, including scavenger function via their respective C-type lectins and antigen-presentation to $CD4^+$ and $CD8^+$ T cells (167). LSECs serve as potent antigen-presenting cells, as they constitutively express MHC Class II molecules on their surface. Unlike dendritic cells however, LSECs modulate T-cell responses to blood-borne antigens via induction of T-cell tolerance (167). Naïve $CD4^+$ T cells primed by LSECs fail to develop into effector Th1 cells, and instead, show a Th0 phenotype characterized by the expression of IL-10, IL-4, and IFNγ upon restimulation (167). Naïve $CD8^+$ T cells primed by LSECs lose the ability to express effector cytokines such as IFNγ and IL-2 following antigen-specific restimulation and also lose their cytotoxic activity against specific target cells (167). Thus, LSECs contribute to antigen-specific T-cell-mediated immune tolerance to blood-

borne antigens. This tolerance induction could contribute to the increased viremia seen in severe dengue disease due to infection of LSECs via L-SIGN and subsequent dampened-host immune responses.

1.4. Differential Neutralization

It is well documented that cell-type-specific glycosylation patterns can alter the antigenic properties of viral proteins (73, 74, 222) and influence the ability of antibodies to neutralize virus (9, 44, 83, 103). Several studies have reported that the peripheral structures of asparagine-linked glycans affect the binding of antibodies to the HIV-1 gp120 envelope (21, 37, 58, 276, 283). Willey *et al.* demonstrated that HIV-1 produced from PBMCs and monocyte-derived macrophages (MDMs) exhibited differential glycosylation of viral envelope that affected viral sensitivities to neutralizing antibodies (283). MDM gp120 contained modifications of the asparagine-linked carbohydrates not observed with the gp120 produced in PBMCs. Neutralization experiments using these isogeneic PBMC and MDM-derived macrophage-tropic HIV-1 isolates indicated that 8 to 10-fold more neutralizing antibody directed against the viral envelope was required to block virus produced from MDM. This neutralization resistance was attributed to the modulation of asparagine-linked glycosylation in MDMs. Similarly, Chackerian *et al.* showed that changes in SIV envelope glycosylation were directly associated with ability to escape neutralization (37). Thus, differential glycosylation due to the cell in which the virus is propagated could result in differential sensitivity of the viruses to neutralization.

Most of the antigenic determinants reactive with antibodies are exposed on the surface of the protein and hence are hydrophilic. Most of the neutralization-resistant variants of flaviviruses obtained so far have amino acid substitutions which cause a change in charge, and it is known that charged residues are important in the interaction of antigenic sites with antibodies (104, 119, 181). An analysis of the E glycoprotein shows that residue E390 is located in a highly hydrophilic region; Asian 390Asn and American 390Asp have the same hydrophilicity value, but Asn is neutral in charge, while Asp is acidic (158). Hiramatsu *et al.* made point mutations in this region, from E383 to E393 (107). Five out of 6 mutants that sustained an amino acid substitution at position 383, 384, or 385 failed to react with dengue-2-specific monoclonal antibody (mAb) 3H5, failed to be neutralized by the mAb, and displayed reduced mouse neurovirulence. Four of the 6 amino acid differences from the dengue 2 sequence involved a change of amino acid charge (107). These results suggest that, in addition to effects of glycosylation on neutralization, the charge of the amino acids at this E390 antigenic site on the virion surface may significantly impact neutralization as well.

1.4.1. Neutralization Assays

The immune response must be tightly regulated between control of the pathogen and damage to the host. This balance can result in partial or incomplete immunity to a pathogen. While there remains an urgent need for vaccines against dengue virus, the theoretic potential for a vaccine to induce a detrimental immune response and enhance a risk for DHF has been a serious impediment to its development. Given that the primary correlate of immunity to dengue is thought to be the presence of neutralizing antibodies

(105, 124, 125, 129), a prerequisite for comparing and optimizing vaccine candidates is the ability to precisely measure the neutralizing antibody responses evoked by vaccines.

The current gold standard for measuring neutralization is the plaque reduction neutralization test (PRNT). Unfortunately, this test is widely recognized to have limitations. Performance is both laborious and time-intensive, as a single assay takes 5-7 days, and the standardization of techniques between laboratories has not yet been established. Further, the PRNT is not amenable to the high-throughput analysis necessary to evaluate large numbers of samples efficiently, and some question its physiological relevance. Previous attempts to develop microneutralization tests for dengue viruses have been reported (120, 206, 274). The enzyme-linked immunosorbent assay (ELISA) format microneutralization tests are based on the assumption that the amount of secreted antigen is directly related to the number of cells infected. However, multiple rounds of infection, cell death and subsequent release of antigen affect the total amount of cell-free antigen measured in culture. In addition, primary virus isolates have varied replication kinetics, resulting in peak antigen expression ranging from three to ten days. Thus, comparing neutralization between viruses is problematic unless the assays take into account viral growth kinetics. Moreover, residual patient antibodies present in the sera must be removed to prevent them from forming complexes with the viral antigen and compete for capture in the ELISA. The numerous washes to prevent this and the various other manipulations required to harvest and measure viral antigen, leads to significant variability of test results.

On the other hand, flow cytometric-based assays in other viral systems have shown that infected cells could be identified by detection of antigen intracellularly (56,

193). These flow cytometric-based assays proved to be highly sensitive and specific for the detection of HIV-1 infected PBMCs and provided quantitative data on the number of cells infected and the inactivation of infectious virus due to reaction with antibody. Development of a flow based assay for dengue virus would allow the high-throughput analysis necessary for evaluating the large number of samples required to determine the role neutralizing antibodies may play in protection and to ascertain at what levels those antibodies are protective.

In addition to the above-mentioned technical limitations of the PRNT assay, results may not be relevant to human dengue infection because the test uses highly passaged, laboratory-adapted viral stocks and dengue susceptible substrates such as African green monkey kidney cells (Vero) or baby hamster kidney (BHK) cells. It has been demonstrated for many different viruses, including dengue, that serial passaging results in numerous mutations and alters cell receptor usage. Furthermore, measuring the reduction of dengue infection in animal renal epithelial cell lines may not be physiologically relevant to determine protection or susceptibility in humans, especially given that those cell types are not thought to be primary infection targets during human disease. Cells of the monocytic lineage are thought to be the principal targets of dengue virus during human infection, with immature dendritic cells as the most permissive (191, 216, 257, 284).

Recent evidence suggests a specialized C-type lectin, DC-SIGN (CD209) on the surface of dendritic cells mediates dengue virus entry and productive infection, with subsequent release of infectious virions (257). DC-SIGN is expressed at higher levels on immature dendritic cells, correlating with the higher levels of infection. Furthermore,

transfection of a human cell line with DC-SIGN renders the otherwise non-permissive cell line highly susceptible to infection. This infection can then be blocked with the addition of monoclonal antibodies against DC-SIGN. Given that DC-SIGN appears to mediate natural infection of human dendritic cells, using a human cell line transfected with DC-SIGN may provide more relevant neutralization results than using animal renal epithelial cell lines.

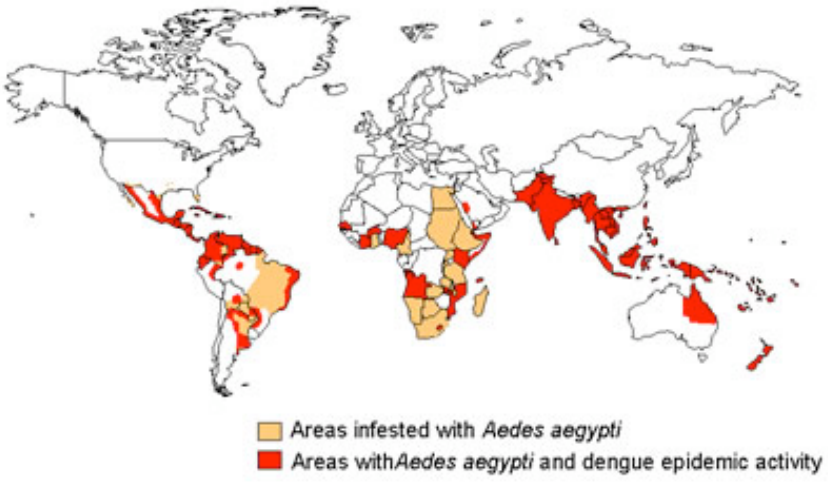


Figure 1-1: World Distribution of dengue viruses and their mosquito vector, Aedes aegypti, in 2005 (WHO, 2005).

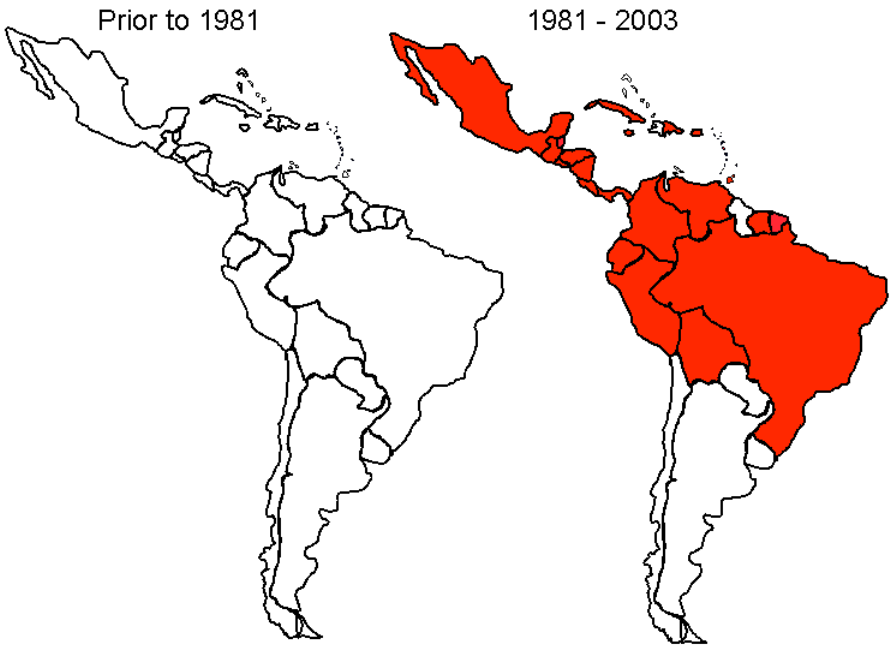


Figure 1-2: American countries with laboratory-confirmed hemorrhagic fever, prior to 1981 and from 1981 to 2003 (WHO/PAHO/CDC, 2004).

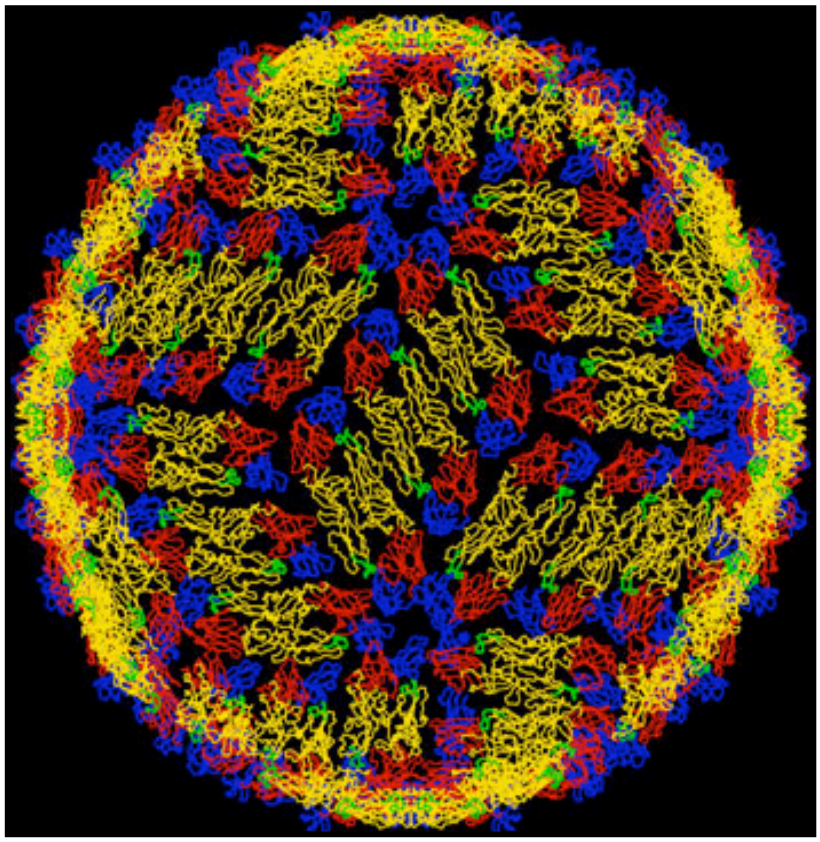


Figure 1-3: Structure of the dengue virion. The envelope glycoprotein forms head-to-tail dimers on the virion surface and each monomer consists of three distinct functional domains named I, II, and III, which are designated red, yellow, and blue, respectively. The fusion peptide is shown in green. (Kuhn, R.J. et al. 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell, 108 (5): 717-25.)

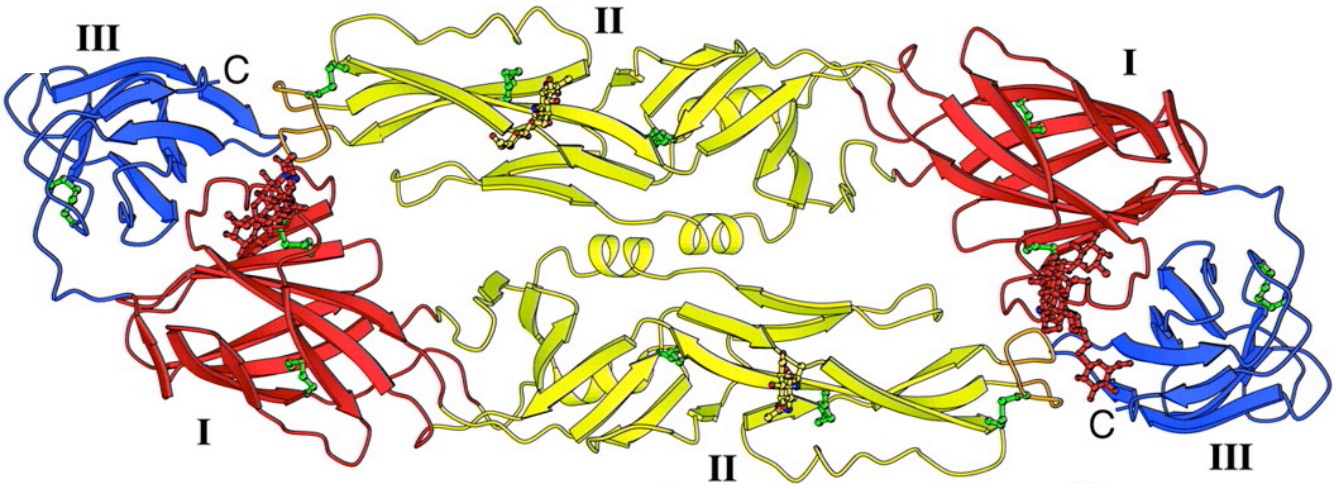


Figure 1-4: Structure of the dimer of dengue envelope glycoprotein in the mature virus particle. The three domains of dengue envelope. Domain I is red, II is yellow, and III is blue. The fusion peptide is in green. (Modis, Y. et al. 2005. Variable surface epitopes in the crystal structure of dengue virus type 3-envelope glycoprotein. J Virol 79 (2): 1223-31.)

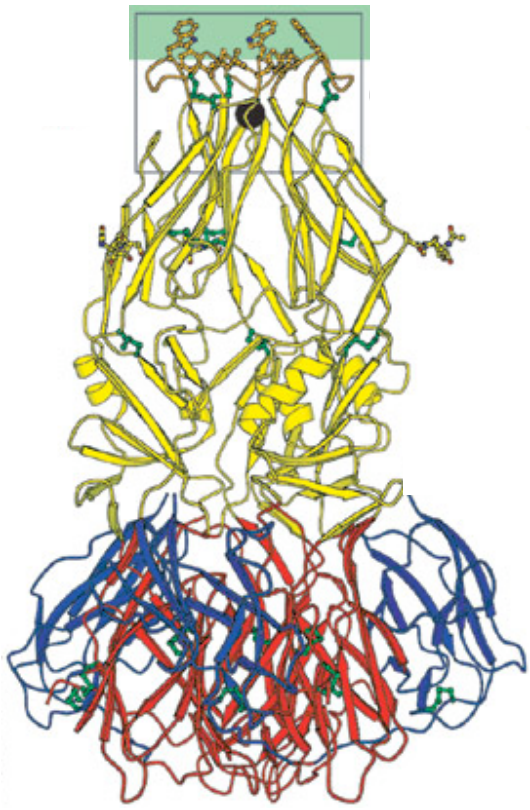


Figure 1-5: The dengue envelope trimer. Ribbon diagram colored as in Fig. 1-4. Hydrophobic residues in the fusion loop (orange) are exposed. The expected position of the hydrocarbon layer of the fused membrane is shown in green. A chloride ion (black sphere) binds near the fusion loop. (Modis, Y. et al. 2004. Structure of the dengue virus envelope protein after membrane fusion. Nature 427 (6972): 313-9).

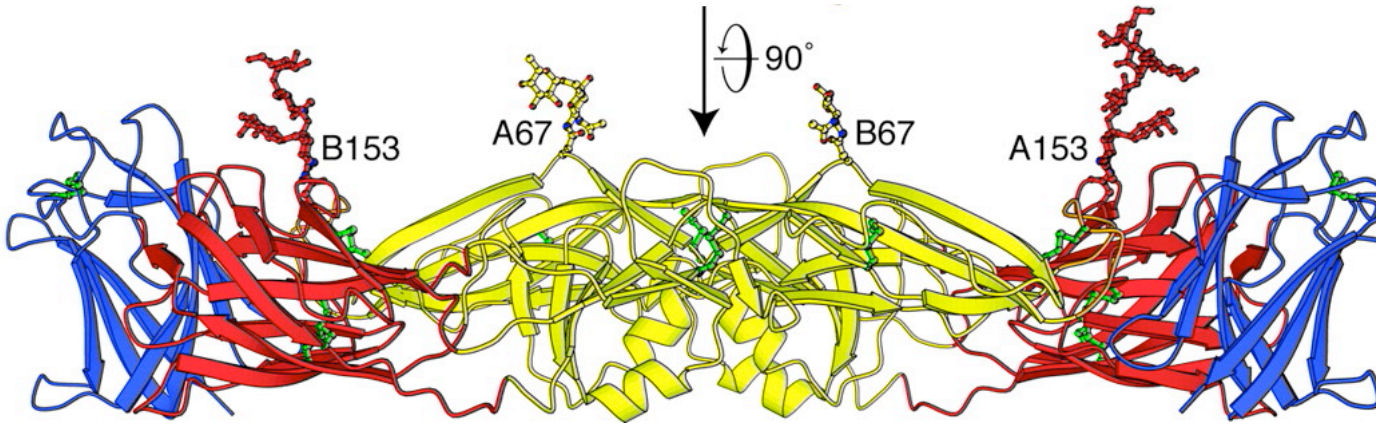


Figure 1-6: Structure of the envelope glycoprotein dimer in the mature virus particle with domains colored as in Fig. 1-4 and viewed 90° perpendicular to its twofold axis. The two glycans on residues 67 and 153 of the two subunits (A and B) of the dimer are labeled. (Modis, Y. et al. 2005. Variable surface epitopes in the crystal structure of dengue virus type 3-envelope glycoprotein. J Virol 79 (2): 1223-31.)

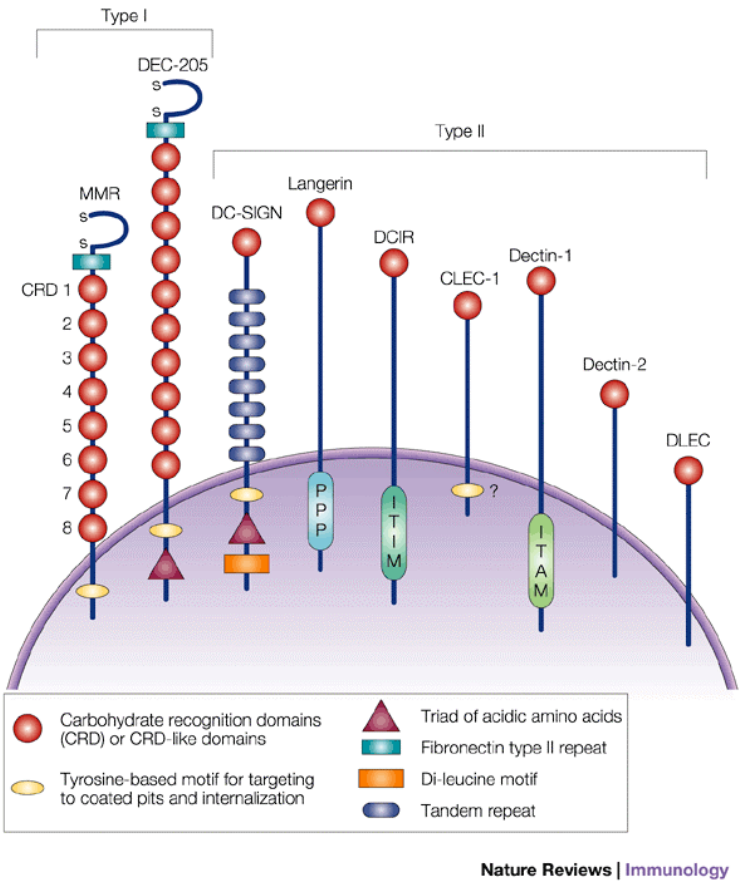


Figure 1-7: Two types of C-type lectins produced by dendritic cells and Langerhans cells. Type I C-type lectins contain an amino-terminal cysteine-rich repeat (S–S), a fibronectin type II repeat (FN) and 8–10 Carbohydrate Recognition Domains (CRDs), which bind ligand in a Ca^{2+}-dependent manner. Type II C-type lectins contain only one CRD at their carboxy-terminal extracellular domain. The cytoplasmic domains of the C-type lectins are diverse and contain several conserved motifs important for antigen uptake: a tyrosine-containing intracellular targeting motif, a triad of acidic amino acids and a dileucine motif. Other type II C-type lectins contain other potential signaling motifs (ITIM, ITAM, proline-rich regions (P). (Figdor, C. G. et al. 2002. C-type lectin receptors on dendritic cells and Langerhans cells. Nat Rev Immunol 2 (2): 77-84)

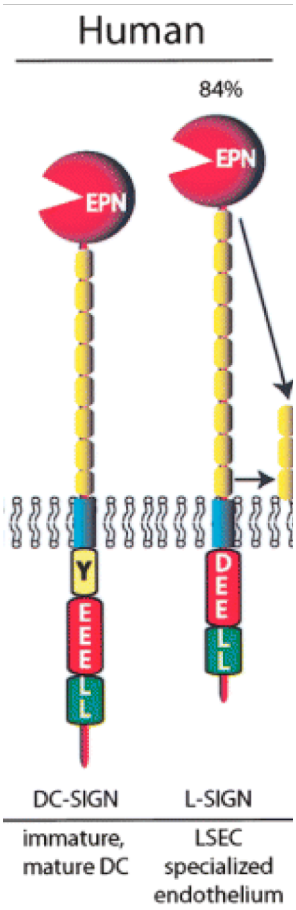
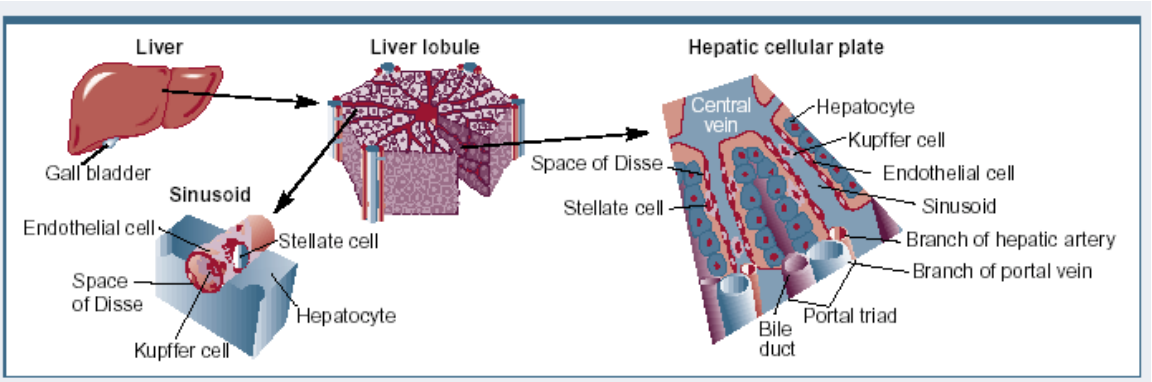


Figure 1-8: Schematic of the structure and expression of DC-SIGN and L-SIGN. Amino-acid-homology is 84%. Within the CRDs, the highly conserved EPN sequence is essential for recognizing mannose-containing structures. DC-SIGN has 7 complete and 1 incomplete repeats, while the number of repeats of L-SIGN varies between 3 and 9. Within the cytoplasmic tail, several internalization motifs are found. The di-leucine (LL) motif is thought to be important for the internalization of DC-SIGN. The tyrosine-based motif and the tri-acidic cluster are also involved in internalization. However, the L-SIGN internalization capacities have not been extensively explored. (Koppel, E. et al. 2005. Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. Cell Microbiol 7 (2): 157-65.)



The liver is situated within the portal triad, an extensive network of blood vessels and bile ducts.

Figure 1-9: Overview of liver structure and function. In addition to hepatocytes, several other cells with special functions are found in the liver. The sinusoidal endothelial cells line the wall of the sinusoids and help clear circulating molecules from the blood. (Friedman, S. 1997. Scarring in alcoholic liver disease: new insights and emerging therapies. Alcohol Research & Health 21 (4): 310).

TABLE 1. Comparison of American and Asian Genotype/Strains of Dengue-2 Virus Envelope Amino Acids.

AA Number	**81**	129	**139**	**162**	203	308	**390**	484
American								
Tonga 74	**Thr**	**Iso**	**Val**	**Val**	**Asp**	**Iso**	**Asp**	**Iso**
Ven2	—	—	—	—	—	—	—	—
IQT 1797	—	—	—	—	—	—	—	—
IQT 2913	—	—	—	—	—	—	—	—
PR152	—	—	—	—	—	—	—	—
PR159	—	—	—	—	—	Val	—	—
131	—	—	—	—	—	—	—	—
539-96	—	—	—	—	—	Val	—	—
Asian								
16681	**Ser**	**Val**	**Iso**	**Iso**	**Asn**	**Val**	**Asn**	**Val**
NGC	—	—	—	—	—	—	—	—
OBS 8035	—	—	—	—	—	Iso	—	—
43	—	—	—	—	—	—	—	—
P7-863	—	—	—	—	—	—	—	—
Philip	—	Iso	—	—	—	—	—	—
Sl77-69	—	—	—	—	Asp	—	—	—
TH-36	—	—	—	—	—	—	—	Iso
Vietnam	—	—	—	—	—	—	—	—

Table 1-1: Comparison of American and Asian genotype/strains of dengue-2 virus envelope amino acids. Representative genotypes listed here geographically span the globe and 60 years of dengue virus isolation. Consistent genetic differences occur between Asian and American and envelope amino acid numbers 81, 139, 162, and 390 (Kochel, T. and Ewing, D., 11/2003).

2. CHAPTER 2: MANUSCRIPT 1

AN IMMUNOCYTOMETRIC ASSAY BASED ON DENGUE INFECTION VIA DC-SIGN PERMITS RAPID MEASUREMENT OF ANTI-DENGUE NEUTRALIZING ANTIBODIES

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ABSTRACT

Dengue remains a global public health threat and development of a safe and effective vaccine is a principal public health goal. The primary correlate of immunity is thought to be neutralizing antibodies. Currently, the plaque reduction neutralization test (PRNT) is the gold standard measure of dengue neutralizing antibody responses, but this test is limited by time-consuming performance. In addition, some feel that use of viral strains adapted to grow in Vero or BHK cells may not accurately reflect protective responses. A human cell line transfected to express a putative natural dengue receptor, DC-SIGN (CD209) was used to measure antibody-mediated dengue neutralization. Using neutralizing monoclonal antibodies, immune sera, and laboratory adapted dengue viruses, serotype-specific neutralizing activity was demonstrated similar to that seen in the Vero PRNT. Importantly, serotype-specific neutralizing activity against recently isolated dengue strains with less heterotypic cross-neutralization than laboratory adapted viruses was also demonstrated.

Keywords: dengue virus, DC-SIGN, neutralization, PRNT, immunocytofluorimetry, dengue antibodies

INTRODUCTION

Dengue constitutes a significant international public health concern, as two-fifths of the world's population live in dengue-endemic regions, and an estimated 50 million cases of dengue infection occur annually(WHO, April, 2002). Belonging to the family *Flaviviridae*, dengue viruses consist of four antigenically distinct serotypes and are transmitted to humans by the bite of infected *Aedes* mosquitoes. Infection may either be asymptomatic or characterized by fever, chills, frontal headache, myalgia, arthralgia, and rash. Subsequent infections with different serotypes may result in more severe manifestations of the disease involving plasma leakage and hemorrhage (dengue hemorrhagic fever) and shock (dengue shock syndrome).

Development of a safe and effective vaccine against dengue infection remains a principal public health goal. Given that the primary correlate of immunity to dengue is thought to be the presence of neutralizing antibodies(Henchal et al., 1988; Kaufman et al., 1989; Kaufman et al., 1987; Kliks et al., 1988), a prerequisite for comparing and optimizing vaccine candidates is the ability to precisely measure the neutralizing antibody responses evoked by vaccines. The current gold standard for measuring neutralization is the plaque reduction neutralization test (PRNT), though this test is recognized to have limitations. Performance is laborious and time-intensive, as a single assay takes 4-7 days, and is not readily amenable to the high-throughput analysis necessary to evaluate large numbers of samples efficiently. Furthermore, the standardization of techniques between laboratories has not yet been established.

A flow cytometric assay capable of determining the presence of neutralizing antibodies to dengue virus within 24 hours was developed. This assay measures dengue

infection in a human cell line transfected to express a dendritic cell pathogen-capturing lectin, DC-SIGN(Tassaneetrithep et al., 2003), which renders the cells highly susceptible to dengue infection. This high-throughput assay is amenable to processing the large number of samples required to delineate the role neutralizing antibodies play in immunity and accurately evaluate vaccination strategies. Similar neutralization activity of specific monoclonal antibodies and sera in the DC-SIGN assay and the conventional PRNT were observed when virus stocks adapted for growth in Vero cells were used as inocula. However, differences in neutralization between recently isolated virus strains and laboratory adapted reference virus strains were found when examined in the DC-SIGN assay using type-specific monoclonal antibodies and sera.

MATERIALS AND METHODS

Cell lines. The DC-SIGN transfected Raji cells, originally developed by Geijtenbeek (initially thought to be THP-1 cells and subsequently identified as Raji cells), were kindly provided by Ralph Steinman (Rockefeller University, NY)(Geijtenbeek et al., 2000; Tassaneetrithep et al., 2003; Wu et al., 2004). These cells were cultured at 37ºC/5% CO_2 in complete medium (cRPMI): RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2mM L-glutamine. The Vero-81 cells used in the plaque reduction neutralization assays were split weekly by trypsinization. They were grown at 37ºC/5% CO_2 in minimum essential medium (EMEM) (Gibco BRL) supplemented with 10% heat inactivated FBS, penicillin G (100 U/ml), streptomycin (100μg/ml), 2mM L-glutamine and 1% sodium bicarbonate. *Aedes albopictus* C6/36 mosquito cells were maintained in Eagle's minimum essential medium

(EMEM; Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100μg/ml), L-glutamine, and nonessential amino acids at 28ºC/5% CO_2.

Virus seed production. The dengue viruses used in this study included the prototypical laboratory adapted viral stocks used in the PRNT in this laboratory: DEN-1 WP74 passage 6, DEN-2 S16803 passage 16, DEN-3 CH53489 passage 15, and DEN-4 341750 Carib passage 12. Strains of all 4 serotypes of dengue isolated in Bandung, Indonesia were used in some experiments, designated DEN-1 BAN98nmrc, DEN-2 BAN98nmrc, DEN-3 BAN98nmrc, DEN-4 BAN98nmrc. The Bandung dengue strains were passaged in cell culture fewer than four times from initial isolation; working stocks were obtained by inoculating a monolayer of C6/36 cells (*Aedes albopictus*) in tissue culture flasks at 28ºC/5% CO_2 for 1 h. After 1 hour, EMEM supplemented with 2% FBS was added and the cells were cultured for 7 days or until greater than 75% of the cells became infected as determined by IFA. Supernatant fluid was harvested and clarified at 5000 rpm for 30 minutes at 4ºC. Virus culture supernatants were aliquoted at 1ml/vial and stored at -80ºC. A limiting dilution plaque assay titration in Vero cells determined the viral titer in plaque forming units per milliliter (PFU/mL).

Vero cell plaque assay. Plaque assays were performed according to the method of Rao (Rao, 1976). Vero cells were seeded into six-well plates at a density of 2.5×10^5 cells/well and incubated for 48 hours. Fifty microliters of dengue virus stock was added to 450μl of maintenance media. The virus mixture was serially diluted using 10-fold dilutions. Two hundred microliters of each dilution of virus was added to each well of

Vero cells in duplicate. The plates were incubated at 37ºC/5% CO_2 for 1 h, rocking plates every 15 min. Three milliliters of primary nutrient agar overlay (1% agarose, EMEM, 5% FBS, 1% non-essential amino acids, 1% penicillin/streptomycin, 0.5% L-glutamine, and 1% HEPES) was added to each well and plates were then incubated at 37ºC/5% CO_2 for 5 days. A secondary saline/agar overlay containing 4% neutral red was added to each well and plates were incubated overnight at 37ºC/5% CO_2 before counting plaques and calculating virus titers. The viral titers were expressed as PFU/ml, calculated as [(number of plaques per well) x (dilution)]/(inoculum volume). These viral titers were used to calculate the MOI in all subsequent experiments for both the PRNT and FACS assay.

Neutralizing antibodies and serum samples. IgG1 mouse monoclonal antibodies (mAbs) 3H5, 4G2, 15F3 were provided by the Walter Reed Army Institute of Research. DEN-2-specific 3H5 and flavivirus group-reactive 4G2 bind to envelope and are known to neutralize, while non-neutralizing 15F3 recognizes NS1(Henchal et al., 1982). Convenience samples of dengue-immune sera from various non-human primate (NHP) and human studies were used in some experiments. Pooled antiDEN-2 sera was obtained from rhesus macaques infected with a prototypical DEN2 serotype. These sera were archived samples, previously collected under protocols approved by the Naval Medical Research Center Institutional Animal Care and Use Committee (IACUC). Sera from human subjects were archived, de-identified samples previously collected from consenting subjects and used in this study under a protocol approved by the Naval Medical Research Center Institutional Review Board (IRB). DEN-2 infection was

determined by virus isolation. All sera were heat inactivated at 56^{O}C for 30 minutes and stored in aliquots at -20ºC prior to use.

Plaque reduction neutralization test (PRNT). The PRNT was performed according to the method of Russell et al. with modifications to use Vero cells (Russell et al., 1967). Heat-inactivated serum specimens were diluted 2-fold (1:40 to 1:1280) with complete EMEM, mixed with an equal volume of virus suspension diluted to give 60-80 plaque-forming units (pfu/well) and incubated for 30 minutes in a 37ºC water bath. This serum-virus mixture (200ul) was inoculated in duplicate onto Vero cell monolayers in 6-well plates and incubated for 1 h at 37ºC/5% CO_2. To each well, 3 ml of enhanced medium (EMEM containing 5% FBS, 1% non-essential amino acids, 0.5% L-glutamine, 1% HEPES, and 1% penicillin/streptomycin plus 1% agar was added. Plates were incubated at 37ºC/5% CO_2 for 5-6 days. Plaques were visualized after addition of saline agar containing 4% neutral red solution and incubation at 37^{O}C for 24 additional hours. A 50% reduction in plaque count was used as the end point of the titration. The percent neutralization was defined as reduction in the number of plaques in the test sera compared with the number of plaques in the control wells with normal sera, at the same dilution. The dilution that neutralized 90%, 80% and 50% (IC90, IC80, IC50) of virus was calculated by curvilinear regression analysis using Prism 4.0 software (GraphPad, San Diego, CA).

Viral infection and FACS analysis for intracellular expression of dengue premembrane (preM) antigen. Dengue infection of DC-SIGN transfected Raji cells

was performed in duplicate in 96 well round-bottomed culture plates by combining 30µl of 5-fold serial dilutions of dengue virus with 60µl of DC-SIGN cells ($1.2x10^5$ cells) and incubated at 37ºC/5% CO_2. After 20 hours, cells were washed in phosphate-buffered saline, fixed and permeabilized using the Cytofix/Cytoperm Kit (BD- PharMingen, San Diego, CA) at 4ºC for 20 min. Permeabilized cells were washed twice in 1X Perm-Wash solution (provided by the manufacturer), resuspended and incubated for 20min at 4ºC with 50µl of a 1:200 dilution of a FITC-conjugated 2H2 monoclonal antibody (recognizes a conserved region of anti-preM among all dengue serotypes) or a goat anti-mouse IgG2a isotypic control antibody. After two additional washes to remove unbound antibody, dengue infected or mock-infected cell samples were acquired from the original 96 well plate (cells were never transferred) using an Automated Microplate Sampler (AMS 96-well plate reader; Cytek, Fremont, CA) attached to a FACScan flow cytometer (Becton Dickinson), and data analysis was performed with Flow Jo software (Tree Star, Inc., San Carlos, CA) on at least 10,000 events. Cells initially gated by forward and side scatter were analyzed for intracellular expression of preM-Ag. The number of preM-Ag positive cells was determined using a bivariate plot of fluorescence versus side scatter; the gate was set on mock-infected cells.

DC-SIGN FACS Neutralization assays. The DC-SIGN FACS neutralization assays were performed in 96 well round-bottom culture plates by incubating 30µl of serial dilutions of test sera and normal sera, in duplicate, and 30µl of dengue virus at a dilution resulting in an infection rate within the linear range of the dose response curve. After incubation for 30 min at 37ºC and 5% CO_2, 60µl of DC-SIGN transfected Raji cells

($1.2x10^5$) was added to each well and the culture was incubated for 20 hours at 37ºC/5% CO_2. To enumerate infected cells, they were washed, fixed and permeabilized, and stained with the 2H2 anti-preM antibody as described above, without transferring the cells from the original plate. Samples were acquired using an Automated Microplate Sampler (AMS 96-well plate reader; Cytek, Fremont, CA) attached to a FACScan flow cytometer (Becton Dickinson). After forward and side scatter gating, at least 10,000 events were counted. Final quantitation of preM-Ag-positive cells was done by subtraction of background events in mock-infected cells (usually less than 10 positives per 10,000 events). The percent neutralization was defined as reduction in the number of preM-Ag-positive cells in the test sera compared with the number in the control wells with normal sera, at the same dilution. The dilution that neutralized 90% and 50% (IC90 and IC50) of virus was calculated by curvilinear regression analysis using Prism 4.0 software (GraphPad, San Diego, CA).

RESULTS

Experiments were conducted to determine if FACS was a sensitive method for detecting dengue virus infection of DC-SIGN transfected Raji cells. DC-SIGN transfected Raji cells and control Raji cells were exposed to dengue virus serotypes 1-4 at an MOI of 0.1 and harvested 20 hours later (Fig. 1). Dengue infected cells were detected using a monoclonal antibody (2H2) directed to viral pre-membrane protein. In this way, only productive infection of cells would be detected by flow cytometric analysis.

Time course experiments were conducted to confirm that the 20-hour time point was sufficient to detect infection via FACS, while still remaining within a single round of

replication. These studies showed that first round peak infection does occur at 20 hours post-infection and accordingly, this time point was chosen for harvesting cells for FACS analysis (data not shown).

Linear dose response between virus input and number of preM-Ag-positive cells. Many virus neutralization studies suggest the same fraction of virus is neutralized per unit time when antibody is in excess regardless of the amount of virus added(Dimmock, 1993; Dulbecco et al., 1956; Mandel, 1978). However, this is only considered true during high antibody excess(Della-Porta and Westaway, 1978; Parren and Burton, 2001; Spouge, 1994). Thus, the accurate measurement of antibody-mediated virus neutralization is best performed in an assay with a linear relationship between the amount of infectious virus and the number of target cells infected(Mascola et al., 2002). In order to test this, DC-SIGN cells were exposed to serial dilutions of virus. In several independent experiments with dengue serotypes 1-4 using both laboratory adapted stock viruses and low passage (less than 4) recently isolated strains, a consistent linear dose response was observed (Fig. 2). Interestingly, the recent isolates required a 10-fold lower viral input to achieve the same infection rate as the laboratory adapted viruses. At high viral inputs, a plateau of infection occurs and the detection of neutralization is overshadowed (data not shown). As the virus is diluted, a linear relationship between virus input and the number of infected cells develops. The MOI at which this occurs depends on the virus serotype and strain used. Based on these results, an MOI that would result in approximately 10% - 20% infection of DC-SIGN cells was chosen, as that infection rate remained in the linear range and permitted clear discrimination of infected

from uninfected cell populations. The linear range was confirmed for each virus prior to use in neutralization assays.

Reproducibility of neutralization measured by the FACS assay. Monoclonal antibodies 3H5, 4G2 and 15F3 were used to measure the inter-assay reproducibility of neutralization of DEN-2 S16803. Serial dilutions of each antibody were used in repetitive experiments. The results from three independent experiments with DEN-2 neutralizing 3H5, flavivirus group neutralizing 4G2 and non-neutralizing 15F3 are shown in Fig. 3. As expected, 3H5 neutralized DEN-2, followed by 4G2, and no neutralization was seen with 15F3. The reproducibility of the results measured by the DC-SIGN FACS neutralization assay was evaluated. When the antibody potency was sufficient to neutralize 90% or more virus infectivity (i.e., 1:40 dilution of 3H5 and 4G2), the coefficient of variation (CV) among experiments was less than 4%. When the non-neutralizing mAb 15F3 was used, non-specific neutralization in the range of 10% could still be reproducibly measured, though the CV was higher (25%). When the potency of the antibody produced 50% virus neutralization (i.e. 1:640 dilution of 3H5) or 20% virus neutralization (i.e. 4G2), the CV among experiments was 3% and 20% respectively.

Reproducibility of serotype-specific neutralization measured by the FACS assay. Serial dilutions of antiDEN-2 rhesus macaque sera were used in repetitive experiments against dengue virus serotypes 1, 2, 3 and 4. The results from three independent experiments are shown in Fig. 4. Only DEN-2 virus was neutralized greater than 50% (meeting the conventionally accepted 50% reduction endpoint (Russell et al., 1967)) at

the serum dilutions tested, demonstrating serotype-specific neutralization. Reduction of DEN-2 virus infection by the antiDEN-2 serum was significantly different from reduction of heterotypic virus infection (F-test, $p<0.0001$). The reproducibility of the results from the DC-SIGN FACS neutralization assay was also determined. When the potency of the antibody produced 97% virus neutralization (i.e. 1:80 dilution against DEN-2 virus), the CV among experiments was less than 1%. When the potency of the antibody produced 33% or less virus neutralization (i.e. serum dilution 1:20 against DEN-1, 3, and 4 virus), the CV among experiments was less than 20%. The 1:1280 dilution resulted in the neutralization of approximately 50% of DEN-2 virus infection and 11%, 6%, and 5% of DEN-1, 3 and 4 serotypes, respectively.

Comparison of neutralization by human sera measured by the FACS assay using both laboratory adapted viruses and recent isolates. Archived samples of human sera previously collected from individuals experiencing natural infections with DEN-2 (confirmed by virus isolation at the time of illness) were tested for neutralizing activity. Serial dilutions of sera were used in at least 3 independent experiments against laboratory adapted dengue virus serotypes 1, 2, 3, and 4. As shown in Fig. 5A, serum collected 6 months after a natural DEN-2 infection significantly neutralized only DEN-2 (F-test, $p<0.0001$), demonstrating serotype-specific neutralization. Fig. 5B shows the neutralizing activity of this same sera against recent viral isolates. Of the 4 isolates, only DEN-2 was significantly neutralized (F-test, $p<0.0001$), demonstrating serotype-specific neutralization similar to those results obtained using laboratory adapted virus, although

the neutralization of recent isolate DEN-2 was slightly more robust than the laboratory adapted DEN-2.

Comparison of the FACS assay and the standard plaque reduction neutralization test. Since most of the published data on dengue neutralization use the plaque reduction neutralization test, several experiments were performed to evaluate if the single-round DC-SIGN FACS assay produced data similar to the PRNT. An archived serum sample from an individual previously vaccinated with a tetravalent dengue vaccine candidate and subsequently challenged with experimental DEN-3 infection was tested. DC-SIGN FACS assay results demonstrate tetravalent neutralization of laboratory adapted dengue viruses, with the highest degree of neutralization against the challenged serotype DEN-3. Using the same laboratory adapted dengue viruses, the PRNT data yielded remarkably similar results (Fig. 6A). The FACS assay results correlated highly with the PRNT data with Pearson correlation R^2 values being 0.91, 0.95, 0.96, and 0.96 for DEN-1, 2, 3, and 4, respectively and $p<0.01$ for each corresponding pair. Fig. 6B shows the neutralization results using this same serum and recent isolates. Unlike the results obtained using laboratory-adapted viruses, only recent isolate DEN-3 was significantly neutralized (F-test, $p<0.0001$). Heterologous cross-neutralization occurred at a much lower level when recent isolates were used as compared to the laboratory adapted dengue viruses.

Convenience samples of sera from 23 rhesus macaques previously inoculated with various DEN-2 vaccines were tested in a standard PRNT and in the FACS assay against the laboratory adapted DEN-2 S16803 virus. Table 1 shows the IC90, IC80, and IC50 values, or the reciprocal dilutions that produced 90, 80, and 50% virus neutralization,

respectively. The PRNT IC90 and IC80 values were consistently higher than the corresponding FACS assay values. However, the IC50 values between the PRNT and FACS assays are remarkably similar. The discrepancy between the serum concentrations required for higher virus neutralization thresholds is presumably related to the difference in virus inocula relative to serum between the two assays. In the conventional PRNT, the MOI and "target cell infection rate" vary for individual viruses but are in the range of 0.0001 to 0.0004, to achieve an infection rate of ~40 plaques, or focus-forming units, per 8×10^5 Vero cells (infection rate = 0.005%); this viral inoculum is used to "challenge" 250-300uL of antibody-containing serum. In contrast, 30uL of serum is tested against an MOI of ~0.01 to 0.1 (depending upon the virus strain) used to achieve the target cell infection rate of ~ 20% for the DC-SIGN assay.

DISCUSSION

Clinical trials of candidate tetravalent dengue vaccines will require the accurate and efficient testing of large numbers of sera for vaccine-induced anti-dengue neutralizing antibody to all four dengue serotypes. Because the PRNT requires four to seven days to complete, it is of limited utility when large numbers of samples must be processed. In some circumstances, a further limitation may be the use of dengue virus strains adapted to replicate in cells used as the substrate for the assay, which do not represent natural replication targets. The first assay developed to measure anti-dengue neutralizing antibody used LLC-MK2 cells derived from rhesus monkey kidney(Russell and Nisalak, 1967; Russell et al., 1967). Morens, *et al*, subsequently reported on the use of baby hamster kidney (BHK) cells in a more simplified assay to measure dengue

neutralizing antibodies (Morens et al., 1985)and more recently, Vero cells (derived from African green monkey kidney cells) were used in neutralization assays(Blaney et al., 2005; Guirakhoo et al., 2004). All three assays have been used to measure neutralizing antibody responses in small numbers of subjects immunized with candidate dengue vaccines. Vaccine licensure will ultimately hinge on one or more pivotal Phase III "efficacy trials" demonstrating protection of vaccine recipients from infection. In addition, regulatory agencies will likely also require valid measures of vaccine immunogenicity. Multiple Phase II immunogenicity studies could be required for selection of appropriate vaccine strategies prior to pivotal Phase III licensing trials, and such studies also depend upon reliable and predictive immunogenicity tests. High-throughput is required since a Phase III trial could involve thousands of volunteers with multiple samples per volunteer. Simply conducting the PRNT assays required to make a decision about protective immunity for such a Phase III trial could take years even assuming a rigorous testing schedule. More importantly, a recent WHO initiative multicenter experience (Robert Putnak, personal communication) demonstrates a lack of precision of the current technology within and between laboratories.

Previous attempts to develop high-throughput microneutralization tests for dengue viruses have been reported (Jirakanjanakit et al., 1997; Vorndam and Beltran, 2002). The enzyme-linked immunosorbent assay (ELISA) format microneutralization tests are based on the assumption that the amount of secreted antigen is directly related to the number of cells infected. However, multiple rounds of infection, cell death and subsequent release of antigen affect the total amount of cell-free antigen measured in culture. In addition, primary virus isolates have varied replication kinetics, affecting peak antigen expression.

On the other hand, flow cytometry based assays demonstrate that HIV-infected cells could be identified by detection of antigen intracellularly (Darden et al., 2000; Mascola et al., 2002). These FACS-based assays proved to be highly sensitive and specific for the detection of HIV-1 infected peripheral blood mononuclear cells and provided quantitative data on the number of cells infected and the inactivation of infectious virus due to reaction with antibody, particularly when infection was measured after a single round of replication. Lambeth and colleagues recently reported on their use of flow cytometry to titrate dengue virus based upon detection of infection in C6/36 cells (Lambeth et al., 2005), similar to an earlier report by Sydow, *et al.* (Sydow et al., 2000), and also showed that virus neutralization could be detected in the FACS assay. These papers demonstrate the utility of immunocytofluorimetric dengue antigen detection as a surrogate for measuring dengue infection, as has been previously reported (Diamond et al., 2000; Tassaneetrithep et al., 2003; Wu et al., 2000).

The importance of the cellular receptor and cell substrate system used to measure functional virus neutralization was shown by Griffin and colleagues in their examination of measles protective immunity (Polack et al., 2003). These authors found that a novel neutralization assay using the marmoset B cell line B95-8, in which measles infection is mediated via SLAM (CD150), the receptor for wild type measles isolates, correlated highly with protection from virus challenge in a macaque model. This was in contrast to a conventional measles PRNT using Vero cells, in which infection is mediated via CD46, the receptor for laboratory adapted measles strains. Utilizing LLC-MK2 cell- and BHK cell-based dengue neutralization assays, recent field studies of naturally acquired dengue infection demonstrated that some volunteers developed symptomatic illnesses despite

having pre-illness neutralizing antibodies directed against the infecting dengue serotype (Endy et al., 2004; Porter et al., 2005). In these instances, the neutralizing antibody titers generated by those *in vitro* assays did not correlate with protection against disease in humans. While the reason for the correlation failure is unclear, it underscores the need to develop a dengue neutralization assay that better reflects *in vivo* neutralizing activity in humans, in particular when assessing the potential effectiveness of vaccine-induced neutralizing antibodies against each of the four serotypes during phase II and III trials of candidate tetravalent dengue vaccines.

The flow cytometry-based dengue neutralization assay reported here utilizes the Raji human cell line transfected to express DC-SIGN, a receptor or coreceptor utilized by all four dengue serotypes (Tassaneetrithep et al., 2003). This receptor is present on immature myeloid dendritic cells and mediates their susceptibility to dengue infection *in vitro*, and these cells are postulated to be a primary target for dengue virus replication in humans (Wu et al., 2000). Thus, incorporating the use of this DC-SIGN-expressing cell line into this assay was a logical approach to designing a high-throughput *in vitro* neutralization assay that may better reflect human *in vivo* neutralization. In light of the observed differences between IC90 and IC80 versus IC50 when determined in the DC-SIGN assay compared to the PRNT, it could be that at these stringent neutralization levels (IC90 and IC80), the PRNT may be more sensitive for detecting neutralizing activity, while the FACS assay may be more specific. It is interesting to speculate that choosing the FACS IC80 as the cutoff point might better reflect protective neutralization activity. Studies using pre-illness sera from symptomatic dengue patients are in progress to compare the results generated by the DC-SIGN FACS neutralization assay with data

generated by conventional neutralization assays to determine if the use of DC-SIGN expressing cells produces results that better correlate with *in vivo* neutralization and protection.

Alternatively, it may be that neutralization of laboratory adapted dengue virus strains does not accurately reflect the levels of neutralizing antibodies against currently circulating wild type viruses. Endy, *et al*, (Endy et al., 2004) showed that pre-illness PRNT values were lower against a recent DEN-3 clinical isolate compared to the values generated using the reference DEN-3 virus. This resulted in a decrease in the number of volunteers (from 51% to 36%) with pre-illness neutralizing antibody directed against the infecting serotype for DEN-3. The authors noted that not all clinical isolates could be tested because some did not grow to sufficient titer for use in the neutralization assay. It remains to be seen whether this phenomenon holds true for Raji DC-SIGN expressing cells, but to date a dengue virus has yet to be found that will not readily infect these cells. In fact, the recent isolates used in this study required a 10-fold lower viral input to achieve the same infection rate as the laboratory adapted viruses.

The DC-SIGN FACS neutralization assay described in this article is both rapid and amenable to automation, making it capable of the high-throughput required for analysis of samples from large vaccine clinical trials. Since the infectivity neutralization read-out is derived from the objective measurement by automated flow cytometry of the fraction of cells expressing viral antigen, the technique is expected to be amenable to validation. The assay also enumerates infected cells by the flow cytometric detection of cells expressing preM-Ag, and is thus a means of immunocytometric viral titration. Compared to the conventional neutralization assay, the DC-SIGN FACS neutralization assay directly identifies infected target cells and provides a precise and reproducible

measurement of antibody-mediated inactivation of infectious virus. In addition, low passage, recent dengue virus isolates were shown to efficiently infect cells via DC-SIGN, which facilitates measurement of serum neutralizing activity against wild type circulating viruses.

ACKNOWLEDGEMENTS

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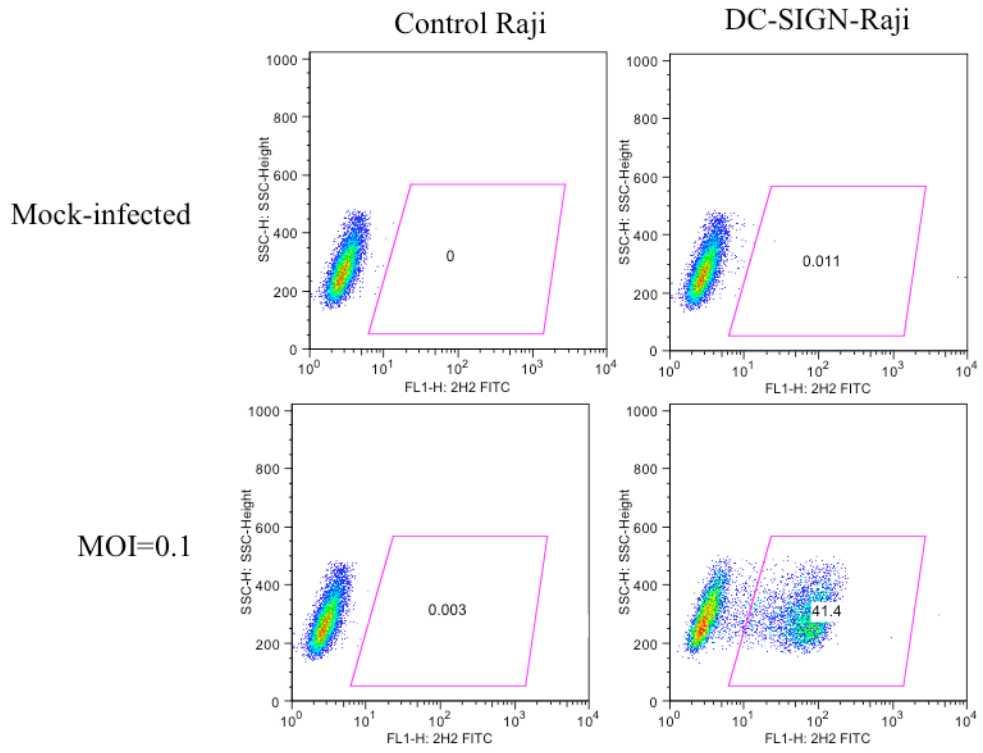
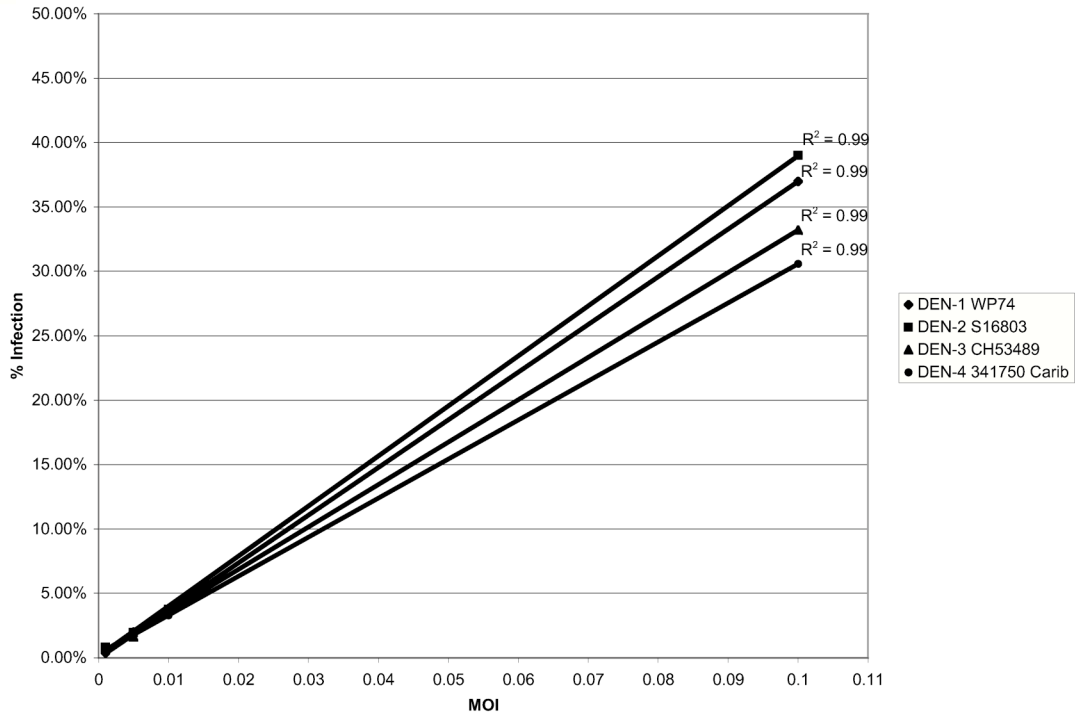


Figure 2-1: FACS data. The Raji cell line, normally resistant to infection, becomes permissive upon DC-SIGN transfection. Flow cytometric scatterplots show the Raji control cell line and Raji cell line transfected with DC-SIGN gated on 2H2-FITC expression (anti-preM-Ag). Both cell lines were exposed to dengue serotypes 1-4 at MOI=0.1 (only DEN2 shown) or mock-infected and harvested for intracellular preM-Ag staining after 20 hours. Raji control cells show background fluorescence of 0.0%, while 0.011% of mock-infected and 41.4% of DC-SIGN transfected Raji cells are positive for intracellular preM-Ag.

A.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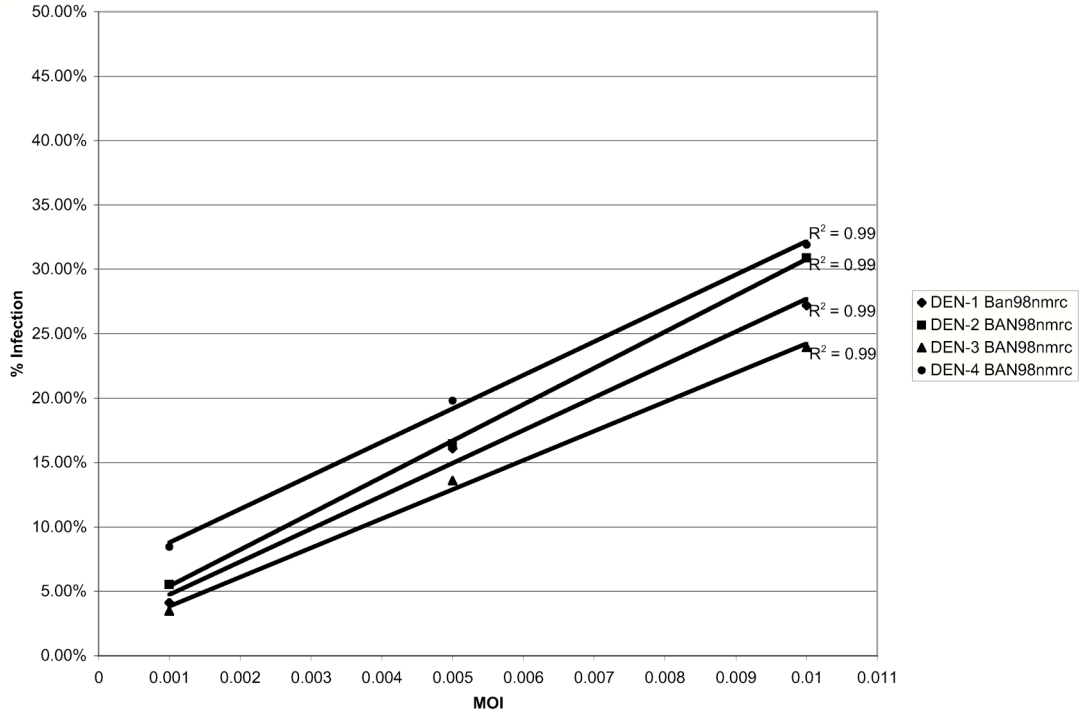
MOI
R² = 0.99
R² = 0.99
R² = 0.99
R² = 0.99
DEN-1 WP74
DEN-2 S16803
DEN-3 CH53489
DEN-4 341750 Carib

B.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R² = 0.99
R² = 0.99
R² = 0.99
DEN-1 Ban98nmrc
DEN-2 BAN98nmrc
DEN-3 BAN98nmrc
DEN-4 BAN98nmrc

Figure 2-2: Linear dose response between virus input and number of preM-Ag-positive cells. (A) DC-SIGN transfected cells were infected with increasing MOIs of laboratory adapted dengue virus serotypes 1-4 (DEN-1 WP74, DEN-2 S16803, DEN-3 CH53489, DEN-4 34170 Carib) or, (B) low passage wild type dengue viruses serotypes 1-4 (DEN-1 BAN98nmrc, DEN-2 BAN98nmrc, DEN-3 BAN98nmrc, DEN04 BAN98nmrc) and incubated for 20hrs. Regression analysis of MOI data demonstrates the relationship.

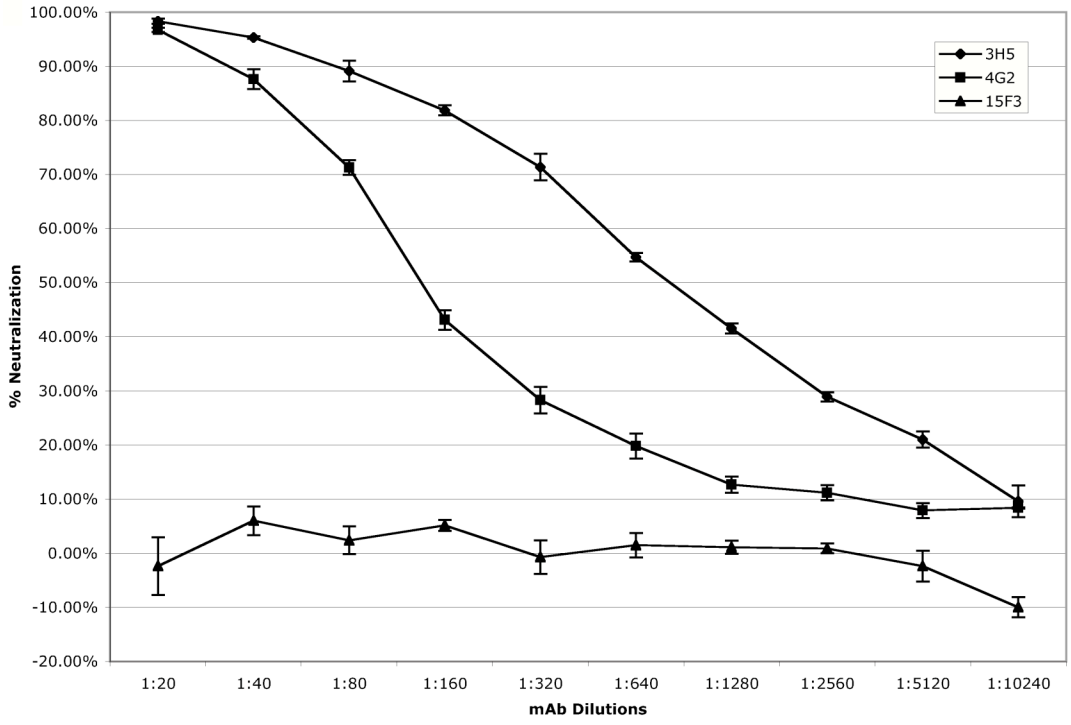

Figure 2-3: DC-SIGN FACS Neutralization assay results using DEN-2 S16803 (MOI=0.1) and monoclonal antibodies (mAb) antiDEN-2 3H5, flavivirus group reactive 4G2, and non-neutralizing 15F3. Each of the neutralization curves is an average (+/- standard error of the mean) of three independent experiments.

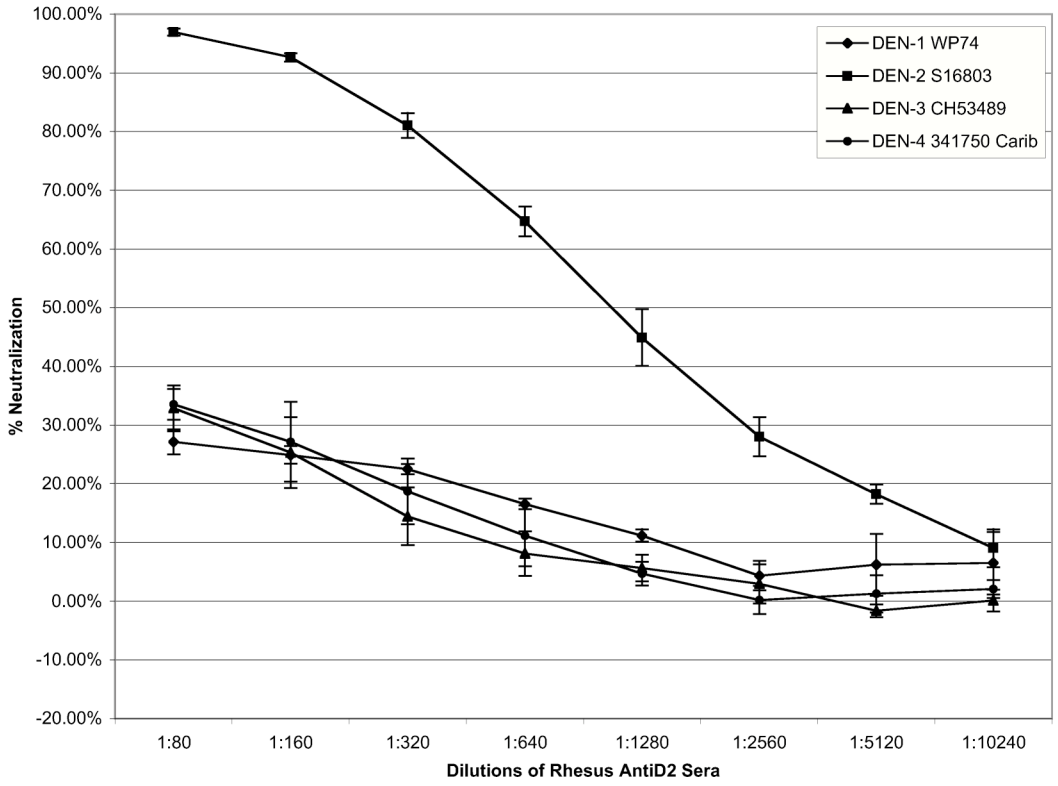

Figure 2-4: DC-SIGN FACS Neutralization assay results using all 4 serotypes of laboratory adapted dengue at MOI= 0.1 and monotypic antiDEN-2 sera from rhesus macaques. Only DEN-2 S16803 met the conventionally accepted 50% reduction threshold, demonstrating serotype-specific neutralization. Each of the neutralization curves is an average (+/- standard error of the mean) of three independent experiments. Significance determined by F-test with $p<0.0001$ for dengue 2 and using Prism 4.0 software (GraphPad, San Diego, CA).

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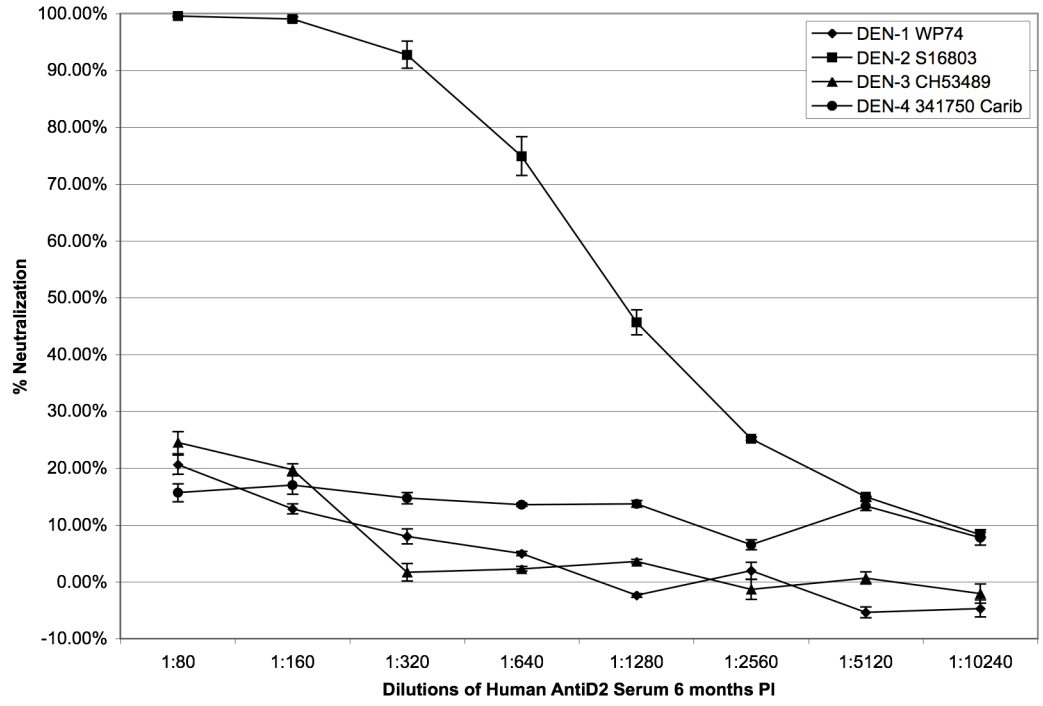
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DEN-3 CH53489
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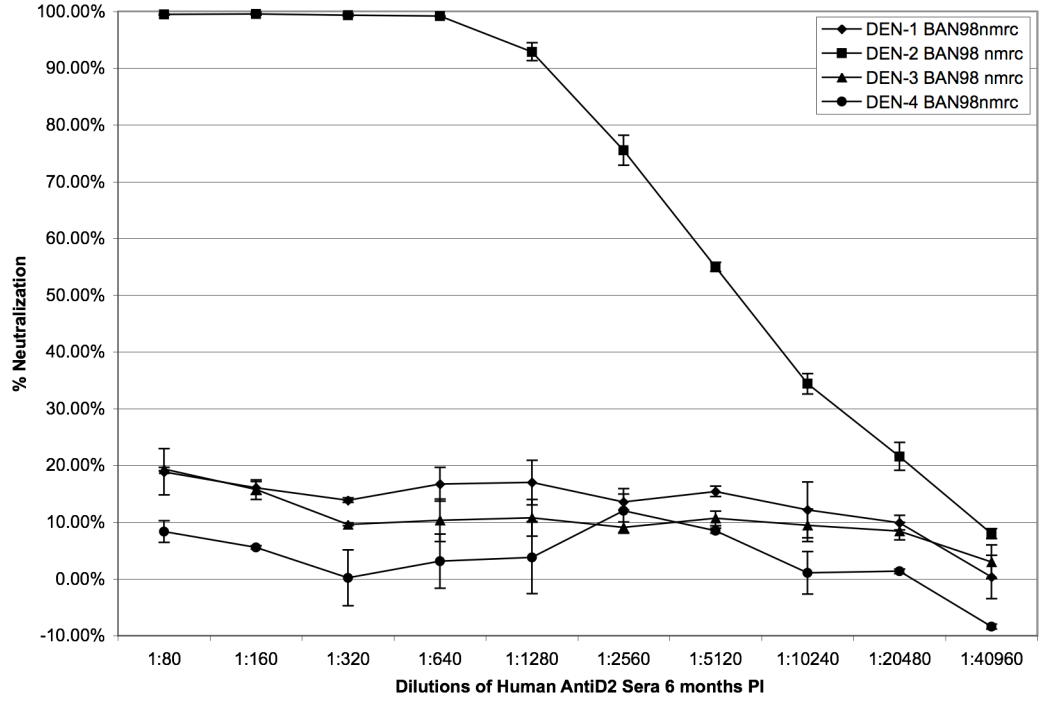
Dilutions of Human AntiD2 Serum 6 months PI

B.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DEN-1 BAN98nmrc
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DEN-4 BAN98nmrc
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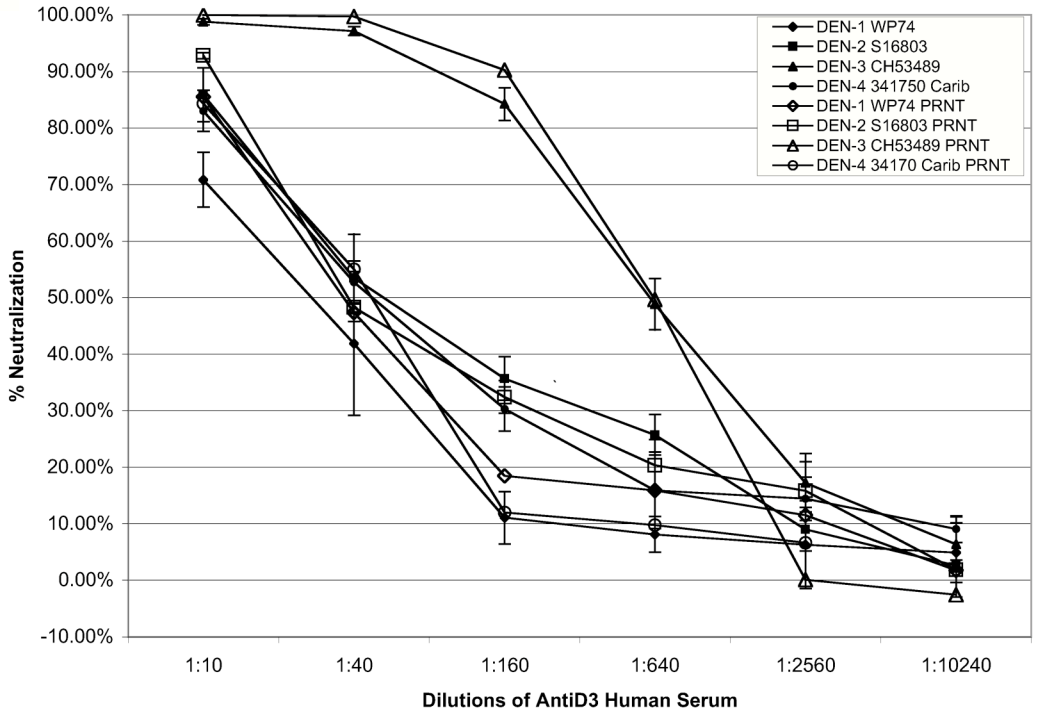
1:20480
1:40960
Dilutions of Human AntiD2 Sera 6 months PI

Figure 2-5: DC-SIGN FACS Neutralization assay results using archived post-infection serum specimens against all 4 serotypes of dengue. (A) Archived serum collected 6 months after a natural DEN-2 infection. Of the 4 laboratory adapted serotypes, only DEN-2 S16803 was neutralized, demonstrating expected serotype-specific neutralization indicative of a matured antibody response. (B) The same convalescent serum specimen was tested against low passage, wild type virus isolates. Only DEN-2 BAN98nmrc was neutralized, demonstrating serotype-specific neutralization with low passage isolates. Each of the neutralization curves is an average (+/- standard error of the mean) of at least three independent experiments. Significance determined by F-test with $p<0.0001$ for dengue 2 and using Prism 4.0 software (GraphPad, San Diego, CA).

A.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% Neutralization
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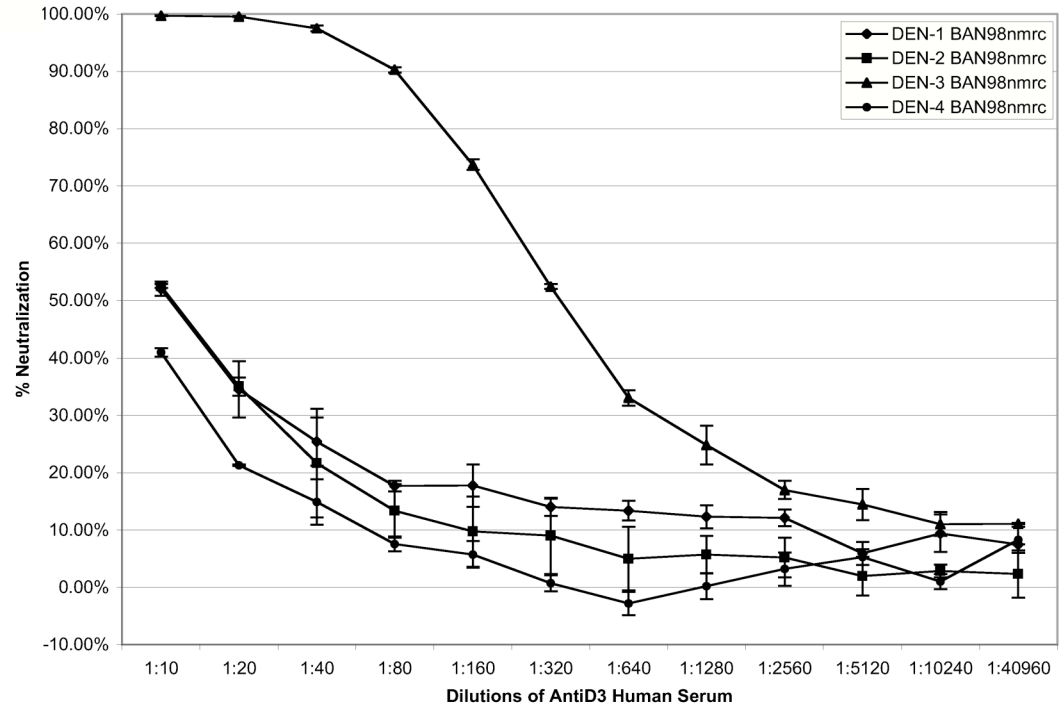
1:2560
1:10240
Dilutions of AntiD3 Human Serum
DEN-1 WP74
DEN-2 S16803
DEN-3 CH53489
DEN-4 341750 Carib
DEN-1 WP74 PRNT
DEN-2 S16803 PRNT
DEN-3 CH53489 PRNT
DEN-4 34170 Carib PRNT

B.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% Neutralization
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1:40
1:80
1:160
1:320
1:640
1:1280
1:2560
1:5120
1:10240
1:40960
Dilutions of AntiD3 Human Serum
DEN-1 BAN98nmrc
DEN-2 BAN98nmrc
DEN-3 BAN98nmrc
DEN-4 BAN98nmrc

Figure 2-6: Neutralization results using all 4 serotypes of dengue and archived serum from an individual previously vaccinated with a tetravalent vaccine candidate collected 60 days after experimental challenge with DEN-3 virus. (A) DC-SIGN FACS Neutralization assay results and PRNT results using all 4 serotypes of laboratory adapted dengue viruses demonstrate similar serotype-specific neutralization with R2 values over 0.91 and $p<0.01$ for each corresponding pair. (B) DC-SIGN FACS Neutralization results using all 4 serotypes of low passage wild type viral isolates. Only DEN-3 BAN98nmrc was neutralized to a significant degree. Cross-neutralization occurred at a much lower level when wild type isolates were used as compared to laboratory adapted dengue viruses. Each of the neutralization curves for the intracellular preM-Ag neutralization assay is an average (+/- standard error of the mean) of three independent experiments. Significance determined by F-test with $p<0.0001$ for dengue 2 and using Prism 4.0 software (GraphPad, San Diego, CA).

TABLE 1. Comparison of the reciprocal dilutions from the PRNT and the DC-SIGN Neutralization Flow Assay[a]

Sample	PRNT			FLOW		
	IC_{90}	IC_{80}	IC_{50}	IC_{90}	IC_{80}	IC_{50}
1	51	81	175	<20	29	159
2	124	174	309	<20	42	311
3	195	292	583	26	58	569
4	400	617	1300	74	230	1791
5	81	128	271	<20	42	290
6	58	84	164	<20	21	131
7	39	59	122	<20	24	111
8	<10	<10	21	<20	20	26
9	126	177	312	<20	54	378
10	58	89	190	<20	28	188
11	114	168	330	22	72	563
12	158	256	607	22	64	616
13	<40	44	76	<20	24	151
14	<40	46	84	<20	<20	123
15	89	109	159	<20	32	150
16	146	195	315	22	55	312
17	<40	42	63	<20	<20	66
18	17	23	41	<20	<20	45
19	<10	<10	<10	<20	<20	<20
20	46	61	98	20	29	179
21	<40	43	66	<20	<20	67
22	52	71	119	<20	29	193
23	67	91	155	<20	28	162

[a] IC_{90}, IC_{80}, and IC_{50} are the reciprocal dilutions that produced 90, 80, and 50% virus neutralization, respectively. Values are averages of three independent experiments for each sample.

Table 2-1: Comparison of the DC-SIGN FACS Neutralization assay and PRNT using DEN-2 S16803 virus and archived samples of sera from 23 rhesus macaques previously inoculated with a DEN-2 vaccine. The mean IC90, IC80, and IC50 values of three independent PRNT and DC-SIGN FACS Neutralization experiments are displayed.

3. CHAPTER 3: MANUSCRIPT 2

DIFFERENCES IN SIGN RECEPTOR UTILIZATION AND NEUTRALIZATION BY AMERICAN AND ASIAN GENOTYPE DENGUE-2 VIRUSES

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ABSTRACT

Consistent genetic differences exist in the envelope glycoproteins between Asian genotype dengue serotype-2 virus (DEN-2) genotypes associated with dengue hemorrhagic fever (DHF) epidemics, and American genotype DEN-2 genotypes associated only with less severe illness, or dengue fever (DF). Since dengue virus infection can be mediated by the C-type lectins DC-SIGN and L-SIGN, we used cells expressing these lectins to examine whether Asian and American genotype DEN-2 viruses exhibit differences in utilization of these two putative receptors. We found subtle but consistent reciprocal differences between the two virus genotypes with regard to *in vitro* infection. American genotype viruses' infection was mediated more efficiently via DC-SIGN than via L-SIGN, while Asian genotypes preferentially infected cells via L-SIGN. A single mutation in the envelope protein of an American virus at amino acid position 390 from aspartic acid (American) to asparagine (Asian) conferred the Asian *in vitro* infection phenotype with regard to lectin utilization. Furthermore, Asian and American genotypes differed in their sensitivity to neutralization. The neutralizing capacity of DEN-2 specific and flavivirus-specific monoclonal antibodies (mAbs) (3H5 and 4G2, respectively) for Asian virus was significantly decreased when infection was measured in L-SIGN bearing cells compared to DC-SIGN bearing cells. We found that sera from DF patients had much greater neutralizing capacity for Asian virus in L-SIGN cells than did sera from patients who progressed to DHF. Further, the magnitude of neutralization of L-SIGN mediated Asian virus infection was inversely associated with disease severity. Considering differential tissue expression between DC-SIGN and L-SIGN, we propose hypothetical mechanisms by which these differences in receptor

utilization and neutralization sensitivity between American and Asian DEN-2 genotypes may contribute to the role viral strain differences play in the pathogenesis of dengue infection.

Keywords: dengue virus, dengue hemorrhagic fever (DHF), DC-SIGN, L-SIGN, neutralization, dengue receptor, dengue pathogenesis

INTRODUCTION

Transmitted to humans by the bite of infected Aedes mosquitoes (Gubler and Rosen, 1976), dengue virus infection may be asymptomatic or manifest as dengue fever (DF), characterized by fever, chills, frontal headache, myalgia, arthralgia, and rash. More severe manifestations of the disease, involving plasma leakage and hemorrhage (dengue hemorrhagic fever-DHF) and shock (dengue shock syndrome-DSS) can also occur in a subset of individuals after resolution of acute fever. Belonging to the Flaviviridae family (Lindenbach, 2001), dengue virus consists of four antigenically distinct serotypes, dengue 1, 2, 3, and 4. The envelope (E) glycoprotein mediates viral attachment and entry by membrane fusion and consists of a homodimer in which each monomer has three β-barrel domains (Zhang et al., 2003). The central structural domain (domain I) contains the N-terminus and is flanked on one side by an elongated dimerization domain (domain II), which contains a fusion peptide at its distal end. Domain III is an immunoglobulin (Ig)-like domain that is thought to contain the putative receptor binding sites (Chen et al., 1997; Hung et al., 1999; Bhardwaj et al., 2001; Crill and Roehrig, 2001; Lee and Lobigs, 2002; Chiu and Yang, 2003).

Genetic analysis has revealed consistent differences in the E glycoprotein between DEN-2 genotypes associated with DHF epidemics (Asian genotype) and DEN-2 genotypes only associated with DF (American genotype) (Rico-Hesse, 1990; Chungue et al., 1993; Deubel et al., 1993; Lewis et al., 1993; Chungue et al., 1995; Rico-Hesse et al., 1997). One of the observed differences in the E glycoprotein between Asian and American DEN-2 genotypes resides in Domain III at residue E390. Whereas Asian genotypes have a neutral asparagine (Asn or N), American genotypes have a negatively

charged aspartic acid (Asp or D). Given that E390 is located in the putative receptor-binding domain (Domain III) (Rey et al., 1995; Chen et al., 1997), a possible functional consequence of substitutions at this position is an altered receptor interaction.

The E glycoprotein also has two potential sites for N-linked glycosylation at positions Asn-67 and Asn-153, which are located spatially near domain III (Rey et al., 1995; Modis et al., 2003; Zhang et al., 2003; Zhang et al., 2004). The number and position of these potentially glycosylated residues is not conserved among different serotypes of dengue virus (Johnson et al., 1994),. C-type lectins, including DC-SIGN (Dendritic-Cell-specific ICAM-3 grabbing non-integrin (DC-SIGN/CD209) and L-SIGN (Liver/lymph node-specific ICAM-3 grabbing non-integrin/CD209L) have been shown to mediate dengue virus infection *in vivo* (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). DC-SIGN and L-SIGN are mannose-specific lectins that are proposed to specifically interact with the carbohydrate residues on the dengue envelope glycoprotein (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). It has been suggested that carbohydrate moieties on the dengue virus surface might modulate the specificity of receptor binding (Navarro-Sanchez et al., 2003). DC-SIGN is expressed by dendritic cells, a presumed relevant target cell in dengue virus infection (Wu et al., 2000). L-SIGN is expressed on various endothelial cells, including liver sinusoidal endothelial cells (LSECs), which are specialized endothelial cells that have been shown to tolerize naïve T cells. Because carbohydrates interact specifically with lectins, minute changes in viral glycoproteins, such as those that exist between Asian and American DEN-2 viruses, could modify their recognition by C-type lectins, and thus affect viral tropism and neutralization sensitivity (Daniels et al., 2002).

We hypothesize that Asian and American genotype DEN-2 viruses have different preferential lectin tropisms. It may be that Asian DEN-2 viruses utilize L-SIGN more than American viruses. The increased viremia seen in DHF patients infected with Asian genotype viruses may be due in part to infection of liver sinusoidal endothelial cells (LSECs), which could occur via L-SIGN. These specialized endothelial cells have been shown to tolerize naïve T cells (Knolle and Gerken, 2000; Limmer et al., 2000; Knolle and Limmer, 2001; Limmer and Knolle, 2001; Knolle and Limmer, 2003). DHF-associated Asian genotypes may preferentially bind L-SIGN, resulting in not only an expansion of replication sites, but also a dampening of the immune response. Furthermore, given that neutralization sensitivity can vary with viral tropism (Mandl et al., 1989; Hasegawa et al., 1992; Jiang et al., 1993; Hiramatsu et al., 1996), an additional potential difference between patients with DHF and those with DF may be the relative paucity of neutralizing activity for L-SIGN-mediated Asian virus infection.

In this study, we used cells expressing DC-SIGN and L-SIGN to examine whether Asian and American genotype DEN-2 viruses exhibit differences in utilization of these two lectin receptors. In addition, we used Tonga 74, a representative American genotype DEN-2 virus with a mutation in E at amino acid 390 to the Asian asparagine amino acid to evaluate the role of that specific envelope change (designated Tonga 390). Since neutralization sensitivity can vary with receptor utilization (Mandl et al., 1989; Hasegawa et al., 1992; Jiang et al., 1993; Hiramatsu et al., 1996), we also examined whether Asian and American DEN-2 genotypes differed in their sensitivity to neutralization by monoclonal antibodies (mAbs) when measured by infection of SIGN-bearing cells. Finally, we used acute serum samples from Venezuelan patients who were diagnosed with DF or DHF in order to examine whether differences in receptor utilization and

neutralization sensitivity may contribute to the role that viral genotype differences play in dengue pathogenesis.

MATERIALS AND METHODS

Cell lines. The DC-SIGN transfected Raji cells were kindly provided by Dr. Ralph Steinman (Rockefeller University, NY) (Geijtenbeek et al., 2000a; Tassaneetrithep et al., 2003; Wu et al., 2004). The L-SIGN transfected Raji cells were a kind gift from Dr. Vineet Kewal-Ramani, NCI, Frederick, MD. These cells were split weekly and cultured at 37ºC/5% CO2 in complete medium (cRPMI): RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2mM L-glutamine.

Virus seed production. The DEN-2 viruses used in this study include American genotypes IQT 2913, IQT 2169, and Asian genotype OBS 8035. The latter genotype was originally isolated in Maracay, Venezuela during the six-year period that the Venezuela sera used in this study were collected and thus is representative of the wild-type circulating virus. The American genotype infectious clone Tonga 74 was kindly provided by Dr. Stephen Whitehead, Laboratory of Infectious Diseases, NIAID, Bethesda, MD. The full-length infectious clone RNA was generated by *in vitro* transcription using the SP6 promoter (Promega, Inc., Madison, WI). A mutant infectious clone containing the Asian mutation at E390 was generated by site-directed mutagenesis using the Quickchange-II kit (Stratagene, Inc., La Jolla, CA) according to manufacturer's instructions. The RNA was transfected into C6/36 (Aedes albopictus) cells via

lipotransfection with DOTAP (Roche, Indianapolis, IN) to obtain viral progeny. The dengue viruses were passaged solely in C6/36 cell culture. IQT 2169 was passaged 6 times, whereas IQT 2913, Tonga 74, Tonga 390, and OBS 8035 were all passaged fewer than three times from initial isolation; working stocks were obtained by inoculating a monolayer of C6/36 cells in tissue culture flasks at 28ºC/5% CO2 for 1 h. After 1 hour, EMEM supplemented with 2% FBS was added and the cells were cultured for 7 days or until greater than 75% of the cells became infected as determined by immunofluorescent assay (IFA). Supernatant fluid was harvested and clarified at 5000 rpm for 30 minutes at 4ºC. Virus culture supernatants were aliquoted at 1ml/vial and stored at -80ºC.

Vero cell plaque assay. Plaque assays were performed according to the method of Rao (Rao, 1976). Vero cells were seeded into six-well plates at a density of 2.5 x 105 cells/well and incubated for 48 hours. Fifty microliters of dengue virus stock was added to 450µl of maintenance media. The virus mixture was serially diluted using 10-fold dilutions. Two hundred microliters of each dilution of virus was added to each well of Vero cells in duplicate. The plates were incubated at 37ºC/5% CO2 for 1 h, rocking plates every 15 min. Three milliliters of primary nutrient agar overlay (1% agarose, EMEM, 5% FBS, 1% non-essential amino acids, 1% penicillin/streptomycin, 0.5% L-glutamine, and 1% HEPES) was added to each well and plates were then incubated at 37ºC/5% CO2 for 5 days. A secondary saline/agar overlay containing 4% neutral red was added to each well and plates were incubated overnight at 37ºC/5% CO2 before counting plaques and calculating virus titers. The viral titers were expressed as PFU/ml, calculated as [(number of plaques per well) x (dilution)]/(inoculum volume).

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). The concentration of viral RNA was estimated by real-time RT-PCR using the TaqMan system (PE Applied Biosystems, Foster City, CA). RNA was extracted from viral stocks, in duplicate, using the Viral RNA kit (QIAGEN, Valencia, CA). Extracted RNA was amplified in a 50ul final reaction volume by using QuantiTech Probe RT-PCR kit (QIAGEN). Each reaction contained the following: 5X One Step Buffer, 5.5mM MgCl2, 10mM dNTPs, 5uM DEN-2C-IF and DEN-2C-IR, 1.25 U of AmpliTaq DNA Polymerase, 12.5 U of Multiscribe reverse transcriptase, and 20 U of RNase inhibitor. PCR primers and probes were designed to target regions of the capsid gene that are strictly conserved among variants of the Southeast (SE) Asian and American genotypes. Amplification was performed using an ABI Prism 7700 Sequence Detection Instrument (PE Applied Biosystems) as follows: 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 60°C for 1 min. RNA copy number was estimated from a standard curve generated by *in vitro* transcribed RNA standards. Viral RNA standards were prepared by amplifying a 196 bp fragment of the DEN-2 genome (Vero-derived 16803 genotype) with primers DEN-2-OF and DEN-2-OR. The resulting PCR product was ligated into pGEM-T using the PGEM-T Easy Vector System (Promega). Plasmid DNA was linearized with EcoRI and RNA transcripts were generated using the T7 promoter Megascript Kit (Ambion, Austin, TX) according to the manufacturer's specifications. The concentration of transcribed RNA was estimated by UV spectrophotometry.

Antibodies and serum samples. Anti-DC-SIGN specific clone 120507, anti-L-SIGN specific clone 120604, and cross-reactive anti-DC/L-SIGN specific clone 120612 mAbs

conjugated to phycoerythrin (PE) were obtained from Chemicon (Temecula, CA) to phenotype the cells. IgG1 mouse mAbs 3H5, 4G2, and 15F3 were used. DEN-2-specific 3H5 and flavivirus group-reactive 4G2 bind to E protein and are known to neutralize, while non-neutralizing 15F3 recognizes dengue virus nonstructural-1 protein (NS1) (Henchal et al., 1982). De-identified, archived acute illness serum samples from 51 individuals diagnosed with DF and 44 individuals diagnosed with DHF from Maracay, Venezuela (according to WHO criteria) were used. Within the six-year period that these sera were being collected, the virus used in this study (Asian genotype OBS 8035) was isolated in Maracay, Venezuela, and thus is representative of the virus causing infections. DEN-2 infection was previously determined by RT-PCR and virus isolation at the time of illness. All sera were heat inactivated at 56ºC for 30 minutes and stored in aliquots at -20ºC prior to use.

Viral infection and FACS analysis for intracellular expression of dengue premembrane (preM) antigen. A range of genome equivalents of Asian (3.9×10^7 – 1.9×10^{10}) and American (9.0×10^8 – 1.8×10^{11}) genotype viruses were used to infect DC-SIGN- and /L-SIGN-transfected Raji cells. Infection was performed in duplicate in 96-well round-bottomed culture plates by combining 30µl of 5-fold serial dilutions of dengue virus with 60µl of SIGN cells (1.2x105 cells) and incubated at 37ºC/5% CO2. After 20 hours, cells were washed in phosphate-buffered saline, fixed and permeabilized using the Cytofix/Cytoperm Kit (BD- PharMingen, San Diego, CA) at 4ºC for 20 min. Permeabilized cells were washed twice in 1X Perm-Wash solution (provided by the manufacturer), resuspended and incubated for 20min at 4ºC with 50µl of a 1:200 dilution of a FITC-conjugated 2H2 mAb (anti-preM) or a goat anti-mouse IgG2a isotypic control

antibody. After two additional washes to remove unbound antibody, dengue infected or mock-infected cell samples were acquired from the original 96-well plate (cells were never transferred from their original plate) using an Automated Microplate Sampler (AMS 96-well plate reader) Cytek, Fremont, CA) attached to a FACScan flow cytometer (Becton Dickinson), and data analysis was performed with Flow Jo software (Tree Star, Inc., San Carlos, CA) on at least 10,000 events. Cells initially gated by forward and side scatter were analyzed for intracellular expression of preM-Ag. The number of preM-Ag positive cells was determined using a bivariate plot of fluorescence versus side scatter; the gate was set on mock-infected cells. The two lectin-dose response curves were compared using curvilinear regression analysis (Prism 4.0 software, GraphPad, San Diego, CA) with an F-test determining statistical significance ($p<0.05$).

DC/L-SIGN FACS neutralization assays. Neutralizing antibodies to DEN virus were measured by a constant serum-varying virus technique. DEN infection of SIGN transfected Raji cells was performed in duplicate in 96-well round-bottom culture plates by incubating 30µl of a 1:20 dilution of test sera or normal sera and 30µl of 2-fold serial dilutions of dengue virus. After incubation for 30 min at 37ºC 5% CO2, 60µl of DC-SIGN or L-SIGN transfected Raji cells (1.2x105) was added to each well and the culture was incubated for 20 hours at 37ºC/5% CO2. To enumerate infected cells, they were washed, fixed and permeabilized, and stained with the 2H2 anti-preM mAb as described above. Final quantitation of preM-Ag-positive cells was done by subtraction of background events in mock-infected cells (usually less than 10 positives per 10,000 events). The LNI50 value is the log neutralization index 50, or the highest viral inoculum

that the fixed low dilution of serum was able to neutralize by 50% compared to normal serum (Spector and Tauraso, 1968).

Statistical analyses. Data were analyzed with Prism 4.0 software (GraphPad, San Diego, CA). Viral infection rates were compared by generating dose response curves, which were then analyzed using curvilinear regression analysis with an F-test to determine a statistically significant difference ($p<0.05$). Neutralization results were calculated by curvilinear regression analysis and the resulting LNI50 values were combined and averaged for each clinical association (DF or DHF). These two group averages were compared by an unpaired t-test. Group sample sizes of 44 and 51 achieve 80% power to detect a difference of 0.39 in group means with 1 standard deviation and a significance level (alpha) of 0.05 using a two-sided two-sample t-test.

RESULTS

We conducted experiments to determine the level of expression of DC-SIGN and L-SIGN on transfected Raji cells. DC-SIGN transfected Raji cells and L-SIGN transfected Raji cells were incubated with anti-DC-SIGN specific clone 120507, anti-L-SIGN specific clone 120604, and cross-reactive anti-DC/L-SIGN specific clone 120612 mAbs. The phenotyping results demonstrate comparable expression of the SIGN lectin receptors in these two types of transfected cells (Fig. 1).

The viruses used in these experiments were characterized by Vero cell titer and genome copy number (Table 1). Time course experiments were conducted to confirm that the 20-hour time point was sufficient to detect infection via FACS while still

remaining within a single round of replication. These studies showed that first round peak infection does occur at 20 hours post-infection and accordingly, this time point was chosen for harvesting cells for FACS analysis (Martin et al., 2006).

Differential Viral Tropism. DC-SIGN and L-SIGN transfected Raji cells were exposed to a range of viral inocula as determined by genome concentration. Fig. 2A shows American genotype IQT 2913 preferentially utilizes DC-SIGN until 11 logs of viral inoculum. In contrast, Asian genotype OBS 8035 had comparatively greater infection of L-SIGN cells between 8.3 and 10 logs of virus. At viral inocula lower than 8.3 logs and higher than 10 logs, a statistically significant difference between lectin utilization could not be discerned. Fig. 2B shows American Tonga 74 preferentially utilizes DC-SIGN similar to its wild-type counterpart IQT 2913. In contrast, Tonga 390 demonstrates preferential L-SIGN utilization like that of wild-type Asian OBS 8035.

Differential Neutralization. Given the observed differences in infection-mediated receptor utilization, we investigated whether American and Asian viruses would have different neutralization profiles dependent upon whether infection was mediated by DC-SIGN or L-SIGN. Monoclonal antibodies (mAb) 3H5, 4G2, and 15F3 (control mAb) were tested for neutralizing activity. Fig. 3A shows that the neutralizing capacity for mAbs 3H5 and 4G2 for American IQT 2913 is the same for both DC-SIGN and L-SIGN. However, as shown in Fig. 3B, the neutralizing capacity of mAbs 3H5 and 4G2 for Asian OBS 8035 is significantly less for L-SIGN cells than DC-SIGN cells.

Differential Neutralization Associated with Disease Outcome. To elucidate the role that antibody-mediated neutralization might play in disease outcome, we used early illness serum samples from 51 Venezuelan patients who were diagnosed with DF and 44 patients diagnosed with DHF using WHO criteria. Fig. 4 illustrates that no difference in the neutralizing capacity of sera from DF and DHF individuals for DC-SIGN-mediated Asian virus infection. However, for L-SIGN-mediated Asian virus infection, sera from DF patients had a strikingly greater neutralizing capacity than sera from patients who progressed to DHF.

Differential Neutralization Associated with Disease Severity. To evaluate whether the neutralizing capacity of the patient serum was associated with disease severity, the patients who progressed to DHF were subdivided by clinical grade. DHF grade I contains 33 serum samples and a one-sided t-test had enough power to determine a significant difference between DC-SIGN and L-SIGN with $p<0.05$. However, DHF grade II contains only 9 samples. Fig. 5 shows that the magnitude of neutralization of L-SIGN mediated Asian infection was inversely associated with disease severity. As the clinical grade of DHF increased, the neutralizing capacity of the serum decreased.

DISCUSSION

Prior studies of dengue viruses have suggested that genetic differences among genotypes can be associated with attenuation, virulence, and epidemic potential (Mandl et al., 1988; Lobigs et al., 1990; Cecilia and Gould, 1991; Pletnev et al., 1993; Chen et al., 1996; Roehrig et al., 1998). The significance of even one amino acid difference in E

glycoproteins has been established and shown to have dramatic effects on viral tropism and virulence *in vitro* or *in vivo* (Mandl et al., 1988; Lobigs et al., 1990; Cecilia and Gould, 1991; Pletnev et al., 1993). The E glycoprotein on the surface of the dengue virion is the dominant viral antigen and mediates virus attachment and fusion (Chen et al., 1996; Roehrig et al., 1998). Genetic analysis has revealed consistent differences in E glycoprotein between DEN-2 genotypes associated with DHF epidemics (Asian genotype) and DEN-2 genotypes only associated with DF (American genotype). Our results show that Asian and American viruses have different preferential receptor tropisms. The Asian genotype, presumed to be more virulent given its association with DHF epidemics, appeared to make preferential use of L-SIGN as an attachment receptor whereas the American genotype exhibited the reverse infection phenotype.

American genotype IQT 2913 preferentially utilized DC-SIGN until 11 logs of viral inoculum, at which point the infection rate reached a plateau and lectin utilization converged, presumably because the cells became saturated with virus. This phenomenon also occurred with Asian genotype OBS 8035 above 10 logs of virus. Interestingly, significant differences in lectin utilization by Asian OBS 8035 also did not occur below a viral inoculum of 8.3 logs where infection rates were less than 10% for both DC-SIGN and L-SIGN. Perhaps this is due to the ability of Asian OBS 8035 to utilize both lectin receptors more efficiently than American IQT 2913, and so at lower infection rates, preferential utilization can not be discerned.

In addition to genome copy number, we characterized the viruses by Vero titer as well. Table 1 shows that American IQT 2913 has a two log difference in the ratio of genome copy number to Vero cell titer than Asian OBS 8035. In other words, there is 10 times the number of IQT genomes despite a 10 log reduction in IQT infectivity for Vero

cells, as compared to OBS. It is possible that the preparation of American IQT 2913 contains a larger portion of defective interfering particles than the Asian OBS 8035 preparation. Defective particles could affect lectin infectivity results by binding to the lectins, blocking entry of infective particles and thereby result in an apparent reduction in infectivity by the American IQT 2913. In fact, American IQT 2913 did have comparatively lower infectivity for both lectins than Asian OBS 8035. However, there still remains a significant lectin receptor preference of IQT 2913 for DC-SIGN, which is exactly opposite that of Asian OBS 8035. It also seems unlikely that defective particles would differ from infective particles in terms of lectin binding. Thus, we believe our differential tropism results are not confounded by differences in amounts of defective particles in viral preparations. In addition, the infectious clone derived viruses Tonga 74 and Tonga 390 further support the validity of lectin preferences between American and Asian DEN-2 genotype viruses. These clone-derived viruses differ only in one amino acid at 390, and so defective particles would not likely confound the infectivity results.

The observed amino acid difference between American and Asian viruses at position E390 has been considered to be of major importance as a determinant of DHF in cases of secondary DEN-2 infection (Salas et al., 1998; Leitmeyer et al., 1999), as E390 is located in the C-terminal domain III, which contains residues implicated as determinants for host range, tropism, and virulence (Rey et al., 1995). Tonga 74, a representative American genotype DEN-2 virus displayed the American infection phenotype. However, changing the amino acid at E390 to the Asian asparagine changed the *in vitro* infection phenotype from preferential DC-SIGN infection to preferential L-SIGN infection, equivalent to that demonstrated by the native Asian virus. Thus, it appears that the single mutation in American genotype Tonga 74 at E390 from American

aspartic acid to Asian asparagine confers the Asian *in vitro* infection phenotype with regard to SIGN lectin interaction.

DC-SIGN is expressed by dermal dendritic cells (Geijtenbeek et al., 2000b), while L-SIGN shares 77% amino acid sequence identity with DC-SIGN (Soilleux et al., 2000) and is expressed by various endothelial cells, including liver sinusoidal endothelial cells (LSECs). LSECs share a variety of unique features with dendritic cells, including scavenger function and antigen-presentation to CD4+ and CD8+ T cells (Limmer and Knolle, 2001). Unlike dendritic cells, however, LSECs modulate T-cell responses to blood-borne antigens via induction of T cell tolerance(Knolle and Gerken, 2000; Limmer et al., 2000; Knolle and Limmer, 2001; Limmer and Knolle, 2001; Knolle and Limmer, 2003).

While the L-SIGN cytoplasmic domain reveals two putative internalization and sorting motifs that may play a role in receptor endocytosis and trafficking, including a dileucine motif, and a triacidic cluster, the DC-SIGN cytoplasmic domain contains an additional incomplete immunoreceptor tyrosine-based activation motif (Engering et al., 2002). The function of some of these motifs has been characterized to some extent, but the kinetics of SIGN internalization and the specific contribution that these motifs make to the process of SIGN-mediated viral transfer have not yet been elucidated. It is possible that the requirements and efficiency of internalization and receptor cycling differ between the two lectin receptors, which could affect viral infectivity. However, potential lectin internalization and cycling differences does not explain the lectin preference results shown here, as the American virus lectin preference was the exact reciprocal of the Asian virus preference.

Our results show that Asian, or DHF-associated genotypes, preferentially bind L-SIGN relative to American, or DF-only associated genotypes. In a human infection, this preference of Asian virus for cells bearing the L-SIGN putative attachment receptor could contribute to the increased viremia seen in severe dengue disease via two potential mechanisms: (1) increased viral replication due to increased infection efficiency in a larger cellular compartment compared with genotypes preferentially using DC-SIGN, expression of which is restricted to dendritic cells, and (2) subsequently dampened immune response to novel epitopes specific to the virus causing secondary infection, mediated by LSEC-induced tolerization of naïve T cells. In fact, Peyrefitte and colleagues recently demonstrated that an LSEC cell line established from primary liver sinusoidal endothelial cells could efficiently replicate a low-passage DEN-2 virus that originated from Southeast Asian genotypes (Peyrefitte et al., 2006). Infection of the LSEC cell line resulted in the upregulation of ICAM-1 and HLA-1 and increased production of inflammatory mediators, both of which encourage contact with T cells and other lymphocytes. Thus, preferential utilization of L-SIGN by the Asian genotype could be a determinant of disease pathogenesis.

Vaughn and coauthors reported that in cases of secondary DEN-2 infections, the magnitude of peak viremia was associated with disease severity which demonstrates the importance of viral replication and viremia in modulating dengue disease (Vaughn et al., 2000). In our experiments, the sera were heat-inactivated; however, dengue antigen potentially present in the sera was not measured, and it may be that sera from DHF patients had a higher viral load and therefore bound to the antibody and depleted available antibody levels. Nevertheless, this effect was not seen in the DC-SIGN cells, as the neutralizing capacity of both the DF and DHF sera were comparable. Alternatively,

this relative lack of neutralizing capacity of sera from DHF patients for L-SIGN-mediated infection could mirror what occurs naturally in a dengue infection, with higher viremia saturating or overwhelming the dengue neutralizing capacity.

Given the specificity with which carbohydrates interact with lectins, the differences between Asian and American genotype DEN-2 viruses may be due to differential glycosylation, which would explain the difference found in lectin utilization. While one would expect both genotypes to be glycosylated identically given their identical passage history and consensus sequences, there are data that suggest differential glycosylation between viral strains despite identical passage history and sequence (Lin et al., 2000). Lin et al. have shown that infection by Ebola virus Zaire could be enhanced by DC-SIGN and L-SIGN to a greater degree than Ebola virus Sudan (Lin et al., 2003). The authors determined that this effect correlated with the fact that Zaire contained more high-mannose N-glycans than Sudan (Feldmann et al., 1994; Lin et al., 2003).

In addition, Smith and Wright found that both potential glycosylation sites, Asn-67 and Asn-153, in the E glycoprotein of DEN 2 NGC were utilized (Smith and Wright, 1985). However, Johnson et al. demonstrated that DEN 2 JAM was glycosylated only at Asn-67 (Johnson et al., 1994). The authors propose that DEN 2 NGC differs from DEN 2 JAM in terms of the number of carbohydrate moieties attached despite the fact that a comparison of 10 amino acid residues on either side of the N-linked asparagine in DEN 2 NGC and JAM revealed no differences surrounding Asn-153. While we did not determine whether Asian and American genotypes were differentially glycosylated, it is possible that dengue virus glycoprotein N-glycan composition could vary between genotypes, and these differences could impact lectin-binding specificity such that either L-SIGN or DC-SIGN could be preferentially targeted. Alternatively, the change from

acidic Asp (American) to neutral Asn (Asian) in E390 could affect binding to lectins because changes in charge can affect conformation and E390 is spatially located next to the glycosylation region on the dimer protein, and also the glycosylation region on a neighboring dimer (Rey et al., 1995; Modis et al., 2003; Zhang et al., 2003; Zhang et al., 2004; Modis et al., 2005).

Mutations that cause a change in charge are also known to be important in the interaction of antigenic sites with antibodies. Analyses of the E glycoprotein show that residue E390 is located in a highly hydrophilic region (Leitmeyer et al., 1999). While Asn and Asp have the same hydrophilicity value, the first is neutral in charge while the latter is acidic. Hiramatsu et al. made a number of point mutations in this region, from E383 to E393. Five out of 6 mutants that sustained an amino acid substitution at position 383, 384, or 385 failed to react with mAb 3H5, failed to be neutralized, and displayed reduced mouse neurovirulence. Four of the 6 amino acid differences from the DEN-2 sequence involved a change of amino acid charge (Hiramatsu et al., 1996). These results suggest that the charge of amino acids at the E390 antigenic site on the virion surface could significantly impact neutralization. Flavivirus group reactive mAb 4G2 binds to an epitope located in the highly conserved fusion peptide region of Domain II (Roehrig et al., 1998). In the native dimer structure, the fusion peptide loop is packed against the other subunit at DI residue Asn-153 (Crill and Chang, 2004). Our results show that the neutralizing capacity of mAbs 3H5 and 4G2 for American virus was the same for DC-SIGN and L-SIGN; however for the Asian virus genotype, which has an asparagine at E390, the neutralizing capacity was significantly less for L-SIGN. These results indicate the importance of the receptor utilized for virus entry when measuring neutralization.

In conclusion, the decreased neutralizing capacity of mAbs for Asian virus when infection depends on L-SIGN utilization and the Asian virus's infection preference for L-SIGN may be one of the determinants for pathogenesis. Our results show that this viral difference in receptor utilization and neutralization sensitivity is associated with disease outcome. We found that one difference between patients who experience DF and those who progress to DHF after infection with the Asian genotype of DEN-2 is the relative paucity of anti-dengue neutralizing activity to specifically prevent L-SIGN-mediated infection. Furthermore, the magnitude of neutralization of L-SIGN mediated Asian virus infection was inversely associated with disease severity. Infection of LSECs via L-SIGN could result in a tolerizing immune response to novel epitopes that might result in less efficient control of viral replication and thus higher viral burden, which has been shown to correlate with severe disease in secondary DEN-2 infections (Vaughn et al., 2000). This difference in receptor utilization and neutralization sensitivity we found may contribute to our understanding of the role viral genotype differences play in dengue pathogenesis.

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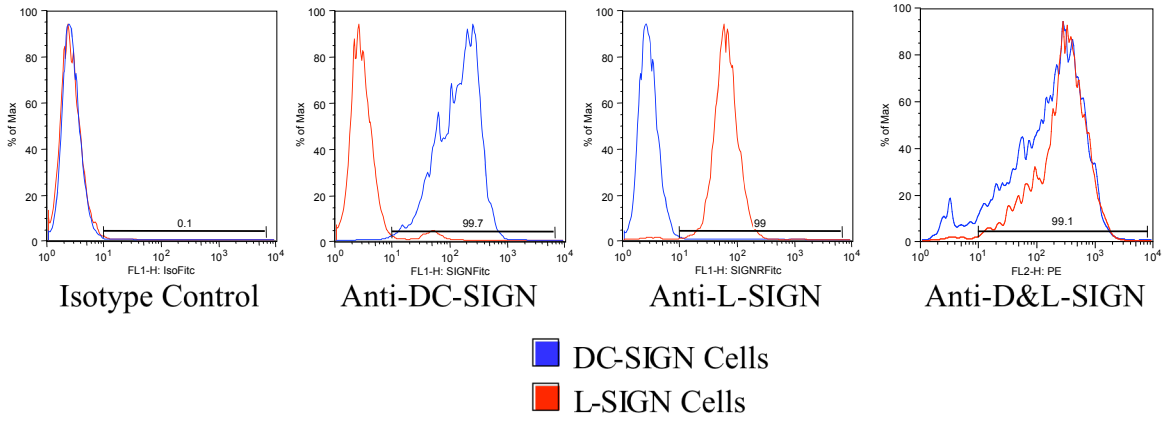
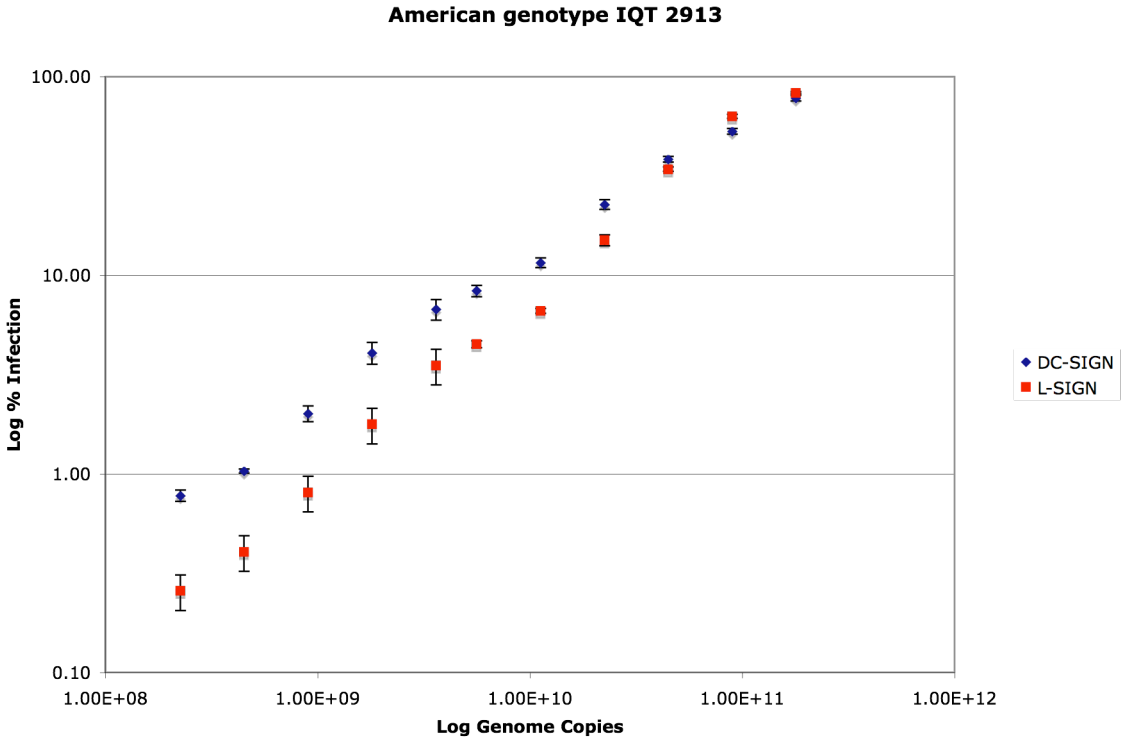


Figure 3-1: Phenotyping results of DC-SIGN and L-SIGN transfected Raji cells. Comparison of receptor expression of DC-SIGN and L-SIGN using isotype matched control, anti-DC-SIGN specific clone 120507, anti-L-SIGN specific clone 120604, and cross-reactive anti-DC-SIGN/anti-L-SIGN specific clone 120612. Unpaired two-sided t-test of geometric MFI of DC-SIGN and L-SIGN expression yielded no significant difference, with p=0.1. Expression of DC-SIGN and L-SIGN are comparable.

A.


American genotype IQT 2913
100.00
10.00
1.00
0.10
Log % Infection
1.00E+08
1.00E+09
1.00E+10
1.00E+11
1.00E+12
Log Genome Copies
DC-SIGN
L-SIGN

B.

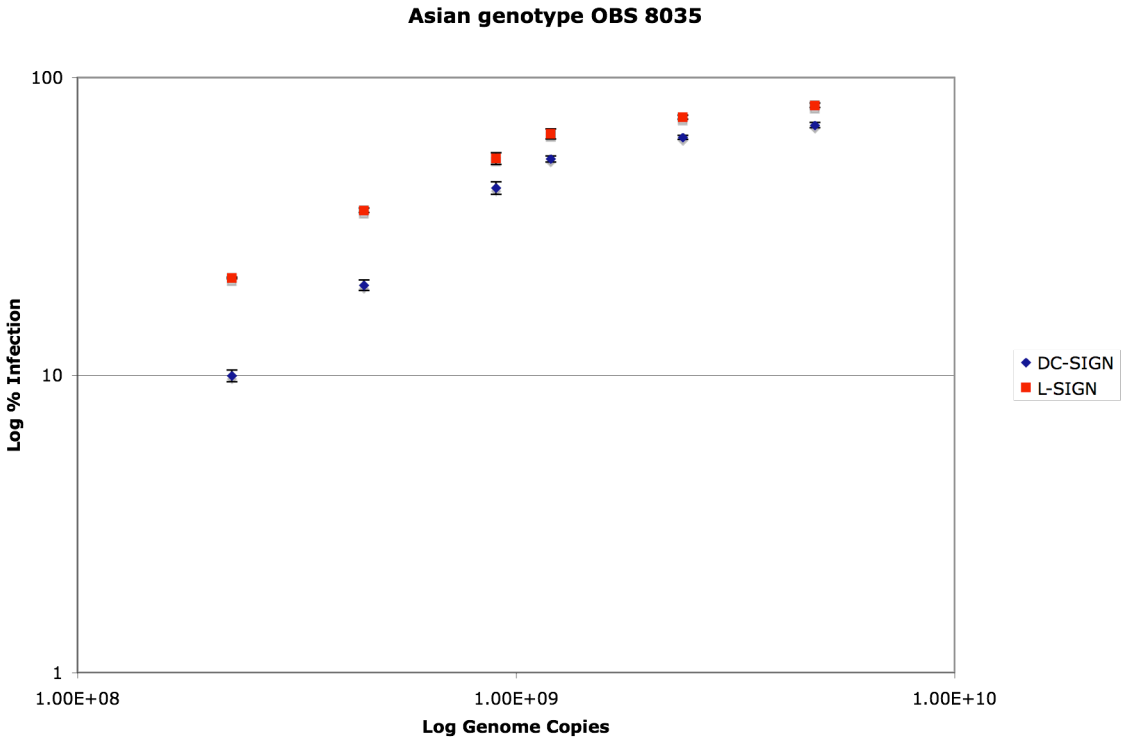
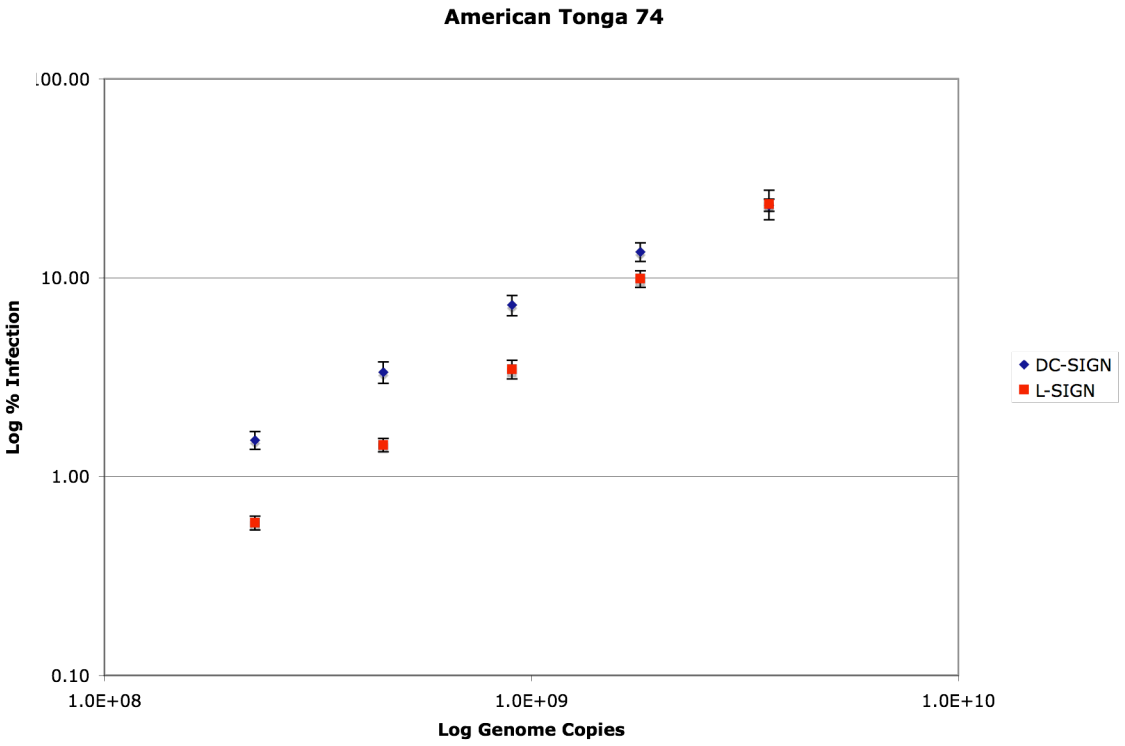

Asian genotype OBS 8035
100
10
1
Log % Infection
1.00E+08
1.00E+09
1.00E+10
Log Genome Copies
DC-SIGN
L-SIGN

Figure 3-2: Differential lectin utilization between American and Asian genotype viruses. Comparison of infection rates of DC-SIGN and L-SIGN bearing cells using a range of viral inocula, as determined by genome copy number. A.) American genotype IQT 2913 preferentially utilized DC-SIGN until an inoculum of 11 logs of virus, at which point lectin utilization converged. B.) Asian genotype OBS 8035 was more efficient at utilizing both lectin receptors than American viruses overall; however Asian virus preferentially utilized L-SIGN at all viral inocula tested. Error bars represent at least 3 independent experiments +/- SEM. Significance determined by curvilinear regression analysis with an F-test ($p<0.05$).

A.


American Tonga 74
100.00
10.00
1.00
0.10
Log % Infection
1.0E+08
1.0E+09
1.0E+10
Log Genome Copies
DC-SIGN
L-SIGN

B.

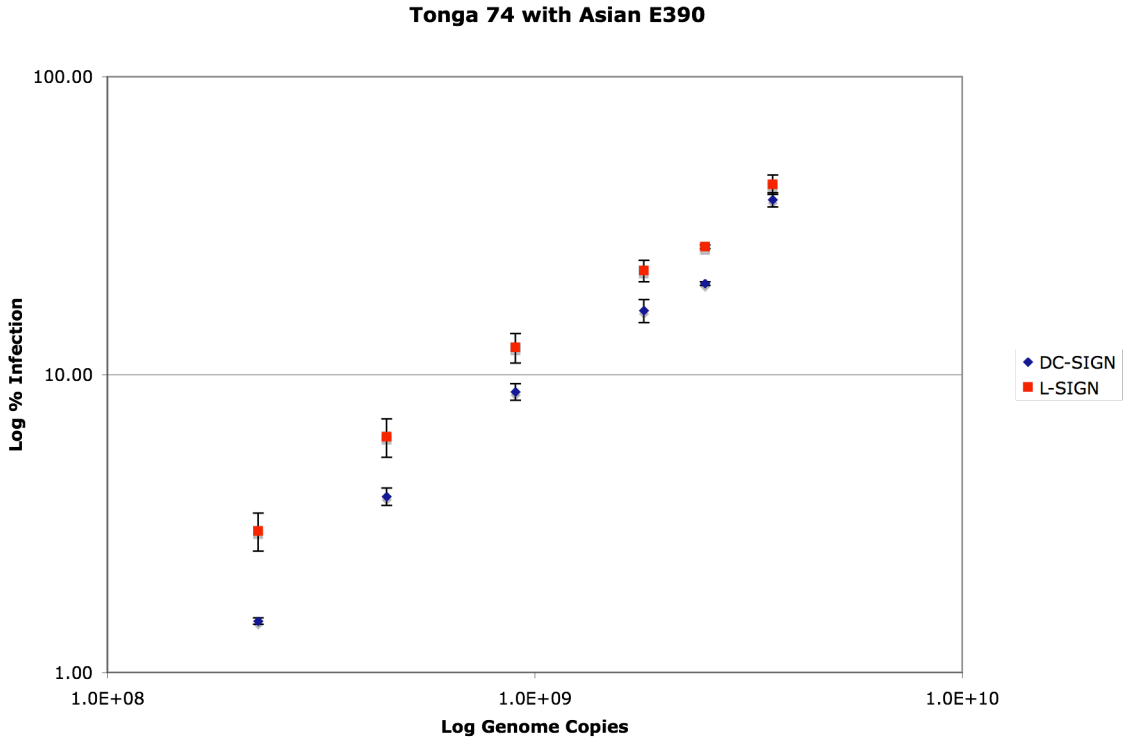

Tonga 74 with Asian E390
100.00
10.00
1.00
Log % Infection
1.0E+08
1.0E+09
1.0E+10
Log Genome Copies
DC-SIGN
L-SIGN

Figure 3-3: Differential lectin utilization between American Tonga 74 and Tonga 74 with Asian E390. Comparison of infection rates of DC-SIGN and L-SIGN bearing cells using a range of viral inocula as determined by genome copy number. A.) American Tonga 74 preferentially utilized DC-SIGN like its wild-type counterpart IQT 2913. B.) Tonga 74 with the Asian E390 preferentially utilized L-SIGN, like the Asian OBS wild-type virus. Error bars represent at least 3 independent experiments +/- SEM. Significance determined by curvilinear regression analysis with an F-test ($p<0.05$).

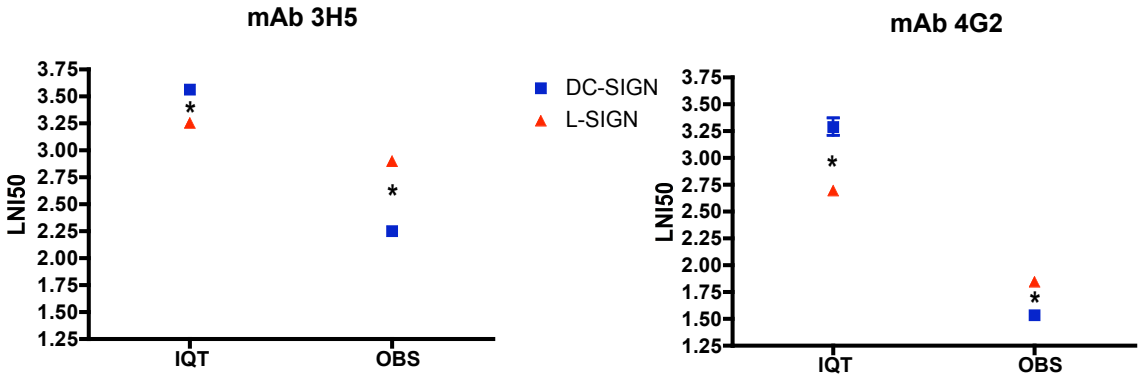


Figure 3-4: Differential Neutralization. A concentration of mAb 3H5 (94ug/ml) and 4G2 (208ug/ml) were incubated with serial dilutions of American IQT 2913 and Asian OBS 8035 dengue 2 viruses. The LNI50 is plotted to compare the neutralization rates between DC-SIGN and L-SIGN bearing cells. The neutralization profiles between DC-SIGN and L-SIGN cells were significantly different for both viruses, as determined by an unpaired two-sided t-test with $p<0.01$. The neutralizing capacity of mAbs 3H5 and 4G2 for DC-SIGN-mediated American and Asian infection was exactly opposite that of L-SIGN-mediated American and Asian infection. The mAbs provided more protection for DC-SIGN cells from American viruses and more protection for L-SIGN cells from Asian viruses. Error bars represent at least 3 independent experiments +/- SEM.

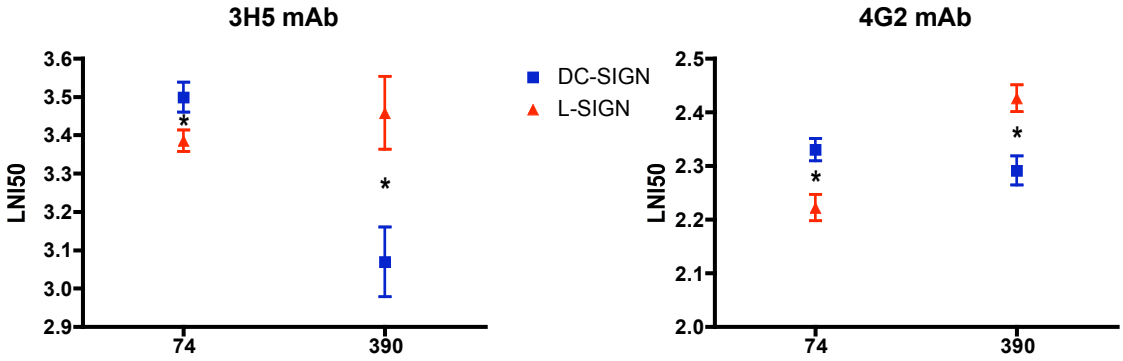


Figure 3-5: Differential Neutralization. A concentration of mAb 3H5 (94ug/ml) and 4G2 (208ug/ml) were incubated with serial dilutions of American Tonga 74 and American Tonga 74 with the Asian mutation at E390. The LNI50 is plotted to compare the neutralization rates between DC-SIGN and L-SIGN bearing cells. The neutralizing capacity of 3H5 and 4G2 is greater for DC-SIGN mediated American infection than for L-SIGN mediated infection. The neutralizing capacity of mAbs 3H5 and 4G2 is greater for L-SIGN mediated Asian infection than DC-SIGN mediated infection. The single mutation at E390 confers the Asian neutralization profile. Significance determined by an unpaired two-sided t-test with $p<0.05$. Error bars represent at least 3 independent experiments +/- SEM.

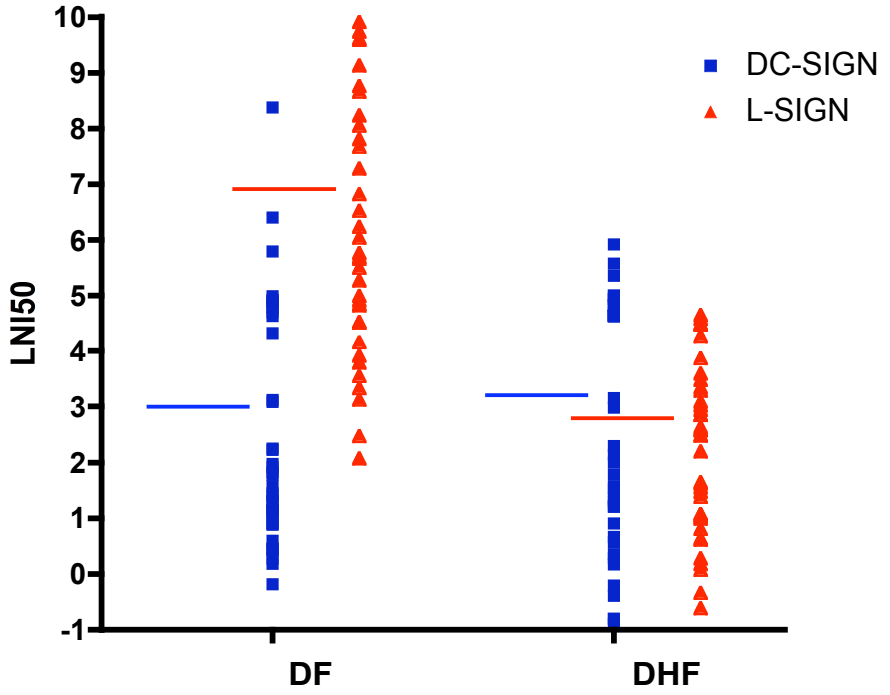


Figure 3-6: Differential Neutralization Associated with Disease Outcome. A constant 1:20 dilution of acute illness serum samples from DF or DHF patients were incubated with serial dilutions of American IQT 2913 and Asian OBS 8035 dengue 2 viruses. The LNI50 is plotted to compare the neutralization rates between DC-SIGN and L-SIGN bearing cells. The neutralizing capacity of acute serum from DF and DHF patients for Asian OBS is comparable for DC-SIGN mediated infection. However, the neutralizing capacity of serum from DF patients is significantly greater for L-SIGN mediated infection. Acute serum from DHF patients lacks this level of neutralizing capacity for OBS and L-SIGN cells. Error bars represent at least 3 independent experiments +/- SEM with $p<0.01$.

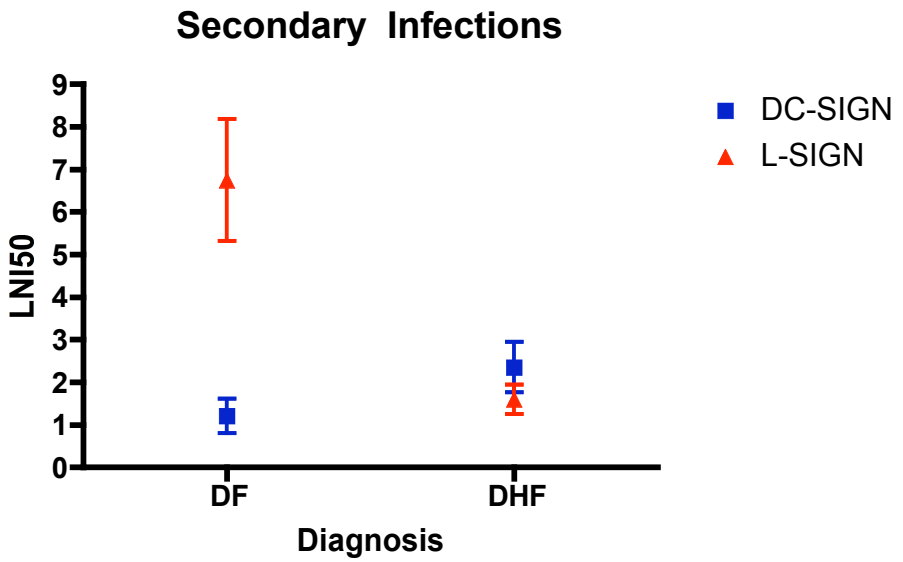


Figure 3-7: Analysis restricted to only confirmed secondary infections (HIA value >2560) Differential Neutralization Associated with Disease Outcome. A constant 1:20 dilution of acute serum samples from DF or DHF patients were incubated with serial dilutions of American IQT 2913 and Asian OBS 8035 dengue 2 viruses. The LNI50 is plotted to compare the neutralization rates between DC-SIGN and L-SIGN bearing cells. The neutralizing capacity of acute serum from DF patients for Asian OBS is significantly greater for L-SIGN bearing cells. Acute serum from DHF patients lacks this level of neutralizing capacity for OBS and L-SIGN cells. Error bars represent at least 3 independent experiments +/- SEM with $p<0.01$.

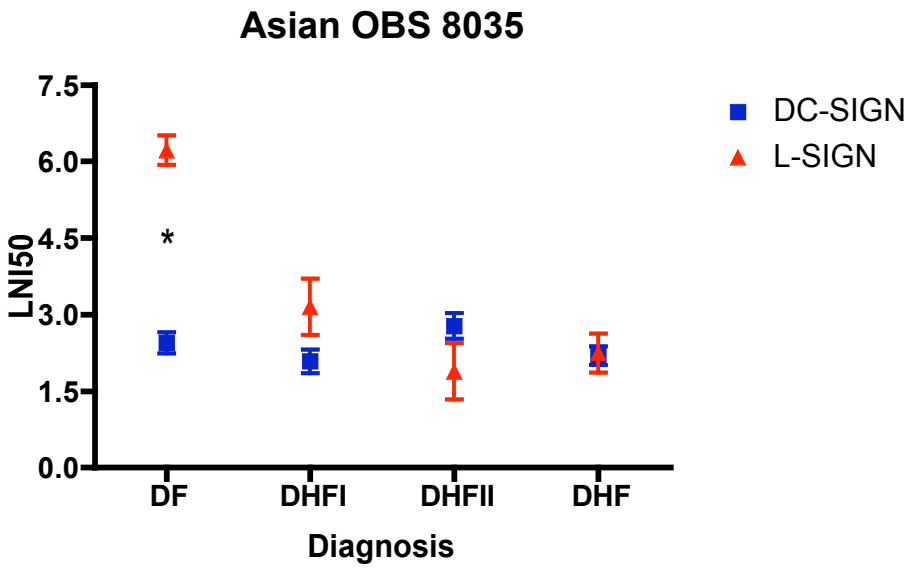


Figure 3-8: Differential Neutralization Associated with Disease Severity. The DHF group was subdivided by clinical grade. The LNI50 is plotted to compare the neutralization rates between DC-SIGN and L-SIGN bearing cells. The magnitude of neutralization of L-SIGN mediated Asian OBS infection was inversely associated with disease severity. Error bars represent at least 3 independent experiments +/- SEM.

4. CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1. Flow Cytometric DC-SIGN Neutralization Assay

Dengue is one of the most important emerging tropical diseases in the 21st century (81, 88, 89). Public health and international agencies have invested little in dengue control, resulting in rapid global spread and major epidemics occurring every 3-5 years (86, 90). Widespread failure of mosquito control programs have further increased efforts to develop tetravalent dengue vaccines (98). Evaluation of candidate tetravalent dengue vaccines will require the accurate and efficient testing of large numbers of sera for vaccine-induced anti-dengue neutralizing antibody to all four dengue serotypes. Several *in vitro* assays using a variety of cell cultures susceptible to dengue infection have been developed. However, the current gold standard assay for measuring the presence of neutralizing antibodies, and therefore evaluating dengue vaccine candidates is the plaque reduction neutralization test (PRNT).

PRNTs measure anti-dengue neutralizing antibody using LLC-MK2 cells derived from rhesus monkey kidney (236, 237), baby hamster kidney (BHK) cells (207) or Vero cells (derived from African green monkey kidney cells) (20, 92). Because PRNTs require four to seven days to complete, they are of limited utility when large numbers of samples

must be processed. Dengue vaccine licensure will ultimately hinge on one or more pivotal Phase III "efficacy trials" demonstrating protection of vaccine recipients from infection. High-throughput will be required since a Phase III trial could involve thousands of volunteers with multiple samples per volunteer. Simply conducting the PRNT assays required to make a decision about protective immunity for such a Phase III trial could take years, even assuming a rigorous testing schedule.

In addition, in order for a particular dengue virus to be amenable for use in these plaque-based readout assays, the virus has to grow to sufficient titer in the cells the assay uses and infection must result in the formation of clear plaques. To facilitate these outcomes, viruses often require adaptation to the cell lines, which is achieved by repeatedly passaging the virus in the cell lines used in the assay. Consequently, viral mutations occur, primarily in the envelope (157) where viral entry is mediated, rendering the virus a more efficient utilizer of the cellular receptors present on those cells. Unfortunately, the receptors the virus becomes adept at utilizing may not be the same receptor the virus uses during the course of a natural human infection. In fact, many in prospective studies the PRNT results using these adapted viruses have failed in many circumstances to predict protection. In other words, PRNT results using pre-infection serum have shown high-titers of antibodies to a certain serotype of dengue, yet those individuals nevertheless get infected and become ill from that dengue serotype. It may be that neutralization of these laboratory-adapted dengue viruses do not accurately reflect the levels of neutralizing antibody against currently circulating wild-type viruses. Interestingly, the DC-SIGN FACS assay results correlated highly with the PRNT data only when using the same laboratory adapted dengue viruses. When recent isolates were used in the DC-SIGN assay, neutralization results differed from the conventional PRNT.

Unlike the kidney cell lines used in current dengue neutralization assays, the flow-based assay uses the Raji human B-cell line transfected to express DC-SIGN, a receptor or coreceptor utilized by all four dengue serotypes (257). This receptor is present on immature myeloid dendritic cells and mediates their susceptibility to dengue infection *in vitro*, and these cells are postulated to be a primary target for dengue virus replication in humans (284). Thus, incorporating the DC-SIGN-expressing cell line into this assay was a logical approach to designing an *in vitro* neutralization assay that may better reflect human *in vivo* neutralization. Using a human cell line expressing a receptor relevant to human infection may also contribute to the elucidation of dengue disease determinants. The flow cytometry-based dengue neutralization assay reported in this thesis also successfully uses low passage, recent dengue virus isolates. Interestingly, infection of DC-SIGN cells by recent isolates required a 10-fold lower viral input to achieve the same infection rate as the laboratory adapted viruses. It may be that using a cell line that expresses a receptor relevant to natural infection allows efficient infection by recent wild type isolates and may reflect more accurate protective responses.

Moreover, since the read-out for the DC-SIGN FACS neutralization assay is derived from the objective measurement by automated flow cytometry of the fraction of cells expressing viral antigen, the technique is expected to be amenable to validation. The DC-SIGN FACS neutralization assay directly identifies infected target cells and provides the precise and reproducible measurement of antibody-mediated neutralization required for valid measures of vaccine immunogenicity. Many parameters needed to be optimized during the development of the FACS neutralization assay in order to obtain precise and reproducible results, including growth kinetics, incubation temperature and time and neutralization kinetics.

Time course experiments were conducted to determine the growth kinetics of dengue virus in both DC-SIGN- and L-SIGN-transfected Raji cells. As with our other experiments, dengue infected cells were detected using a mAb (2H2) directed to viral pre-membrane protein. In this way, only productive infection of cells would be detected by flow cytometric analysis. At time points 0- and 6-hours, no dengue replication could be detected by the FACS readout (Figure 4-1). From 12-hours to 18-hours, replication occurred linearly. At around 20-hours, dengue viral replication reached a plateau and after 24-hours replication increased exponentially, as multiple rounds of replication ensued. Because we were interested in viral entry events, we wanted to harvest experiments after the first round of replication occurred. In order to ensure a strong signal with only one round of replication, we chose the 20-hour time point to harvest all cultures of subsequent experiments.

The optimal temperature to incubate virus with antibody prior to the addition of cells was also determined. As neutralization kinetics would dictate, more neutralization occurred at 37°C than 4°C, thus 37°C was chosen for future experiments as this incubation temperature increased the assay's sensitivity (Figure 4-2). The optimal length of time to incubate virus, antibody and cells was also established (Figure 4-3). We found that after 24 hours of incubation, a neutralization curve could be established. However, after 48 hours of incubation, no neutralization could be detected. We recognized that neutralization results are dependent upon viral kinetics and SIGN-transfected Raji cells were so amenable to dengue viral replication that after 48 hours, multiple rounds of replication masked any initial neutralization results.

To further explore the parameters of neutralization kinetics, we ascertained the extent to which neutralization results could be affected by varying the quantity of viral

inoculum. As expected, we found that as the viral inoculum is decreased, neutralization curves shift to the right (i.e. more neutralization occurs) (Figure 4-4). This neutralization shift occurs presumably because, as the amount of virus is decreased, the ratio of antibodies to virions increases. Interestingly, we discovered that once antibodies are in excess, the neutralization results become much more robust and less sensitive to changes in viral inocula. This occurs only when the viral infection rate is within the linear range. Thus, the linear range of infection for each virus was always determined prior to use (Figure 2-2).

In summary, the DC-SIGN FACS neutralization assay described in this dissertation uses a human cell line transfected to express a putative natural dengue receptor, DC-SIGN. Using prototype laboratory adapted viruses, serotype-specific neutralizing activity results were similar to the Vero PRNT. Interestingly, when low passage, wild-type isolates were used, DC-SIGN results differed from the PRNT, which may suggest the DC-SIGN FACS assay would more accurately reflect levels of neutralizing antibodies in a population against currently circulating viruses, a requirement that the PRNT has been unable to satisfy. The DC-SIGN assay is also both rapid and amenable to automation, rendering it capable of the high-throughput required for analysis of samples from large vaccine clinical trials and prospective studies. Reproducible, precise neutralization results can be obtained within 24 hours as opposed to the 5-7 days required of the standard PRNT assays. In addition to the DC-SIGN transfected human cell line, the FACS neutralization assay can also be used with an L-SIGN transfected human cell line. In this manner, infection rates of different viruses, including multiple strains of dengue 2 can be compared and neutralization patterns of different sera, such as

those from patients diagnosed with dengue fever or dengue hemorrhagic fever, can be studied in order to tease out potential mechanisms of dengue pathogenesis.

4.2. Differential Lectin Tropism and Neutralization

The initial interaction between a virus and its host cell is a critical determinant of viral tropism and pathogenicity; however, little progress has been made in identifying the cells dengue replicates in and the receptor(s) dengue uses to gain entry into those cells. Dengue viruses may employ multiple receptors, only some of which are dengue virus-specific. Non-specific receptors may only serve to bind large amounts of virus on the surface of host cells, facilitating its contact with the dengue virus-specific receptor. Unfortunately, dengue virus-specific receptors have yet to be identified, but most likely interact with domain III of the E protein.

Contributing to the difficulty of identifying a dengue specific receptor is the fact that a wide range of cell types from multiple species is susceptible to infection with dengue viruses. Numerous studies have attempted to identify the cell surface receptor or receptors utilized by dengue viruses to gain entry into susceptible cells, but multiple approaches using different cell lines and virus strains have generated many candidate proteins identified in some cases only by molecular mass (19, 113, 192, 205, 223, 225, 241, 261, 262, 284, 285). Evidence from *in vitro* studies suggest a large variety of cell-types (e.g., hepatocytes, B and T lymphocytes, endothelial cells, monocytes/macrophages, and dendritic cells) could be potential targets for virus infection

and replication, but relatively little is known about the involvement of many of these cells in *in vivo* infections (5, 8, 22, 30, 111, 127, 148, 149, 174, 185, 197, 259).

Human autopsy studies obtained from patients with dengue hemorrhagic fever/dengue shock syndrome revealed the presence of dengue viral antigens in many tissues, including thymus, lymph nodes, kidney, lung (vascular endothelial cells), reticuloendothelial cells, liver (Kupffer and sinusoidal endothelial cells), skin, but mainly in mononuclear phagocytic cells (17, 23, 96, 198),(16, 17, 71, 96). The mere presence of viral antigens within cells, especially phagocytic cells, does not necessarily mean that these cells support viral replication, as the viral antigens may have been phagocytized, killed, or sequestered in immune complexes in the process of being degraded.

However, studies that localize viral RNA using in situ hybridization and reverse-transcription-PCR (RT-PCR) have discovered substantial quantities of viral RNA in the liver, lymph nodes, spleen and in monocytes (118, 232). Two studies using in situ hybridization to detect negative strand viral RNA, indicative of *in vivo* dengue viral replication, found RNA in the thymus, hepatocytes, macrophages in skin and lymph nodes, and peripheral lymphocytes (122, 127). Unfortunately, there are only two of these studies and each was based on only a few cases of dengue infection and a limited range of tissue types. Further investigation is necessary to determine all the locations of dengue virus infection and replication.

One cell type that has been consistently identified by both *in vitro* and *in vivo* studies as supporting dengue viral replication and is currently considered the preferential target for dengue virus infections is human monocyte-derived dendritic cells (152, 191, 216, 239, 284). Human monocyte-derived dendritic cells express the C-type lectin DC-SIGN (Dendritic cell specific ICAM-3 grabbing non-integrin). DC-SIGN is considered to

be the receptor mediating dengue virus infection of dendritic cells, as infection correlates with DC-SIGN expression and non-permissive cells have been rendered susceptible to infection when transfected with DC-SIGN or the DC-SIGN related L-SIGN (Figure 4-5 and Figure 4-6), and this infection can be blocked by specific anti-DC-SIGN antibodies (210, 257). All four dengue serotypes, both lab-adapted and low-passage isolates, can infect dendritic cells via DC-SIGN or L-SIGN (210, 257)

In addition to dendritic cells, several lines of evidence, including case reports, histopathological findings, *in vitro* experiments, and autopsy studies suggest the liver is one of the major target organs of dengue infection and that dengue virus directly invades and replicates in the liver (31, 50, 80, 96, 116, 154, 179, 202, 209, 217, 218, 233, 251, 254, 271, 272, 275). Rosen and colleagues tested autopsy tissues from 18 children with fatal dengue hemorrhagic fever for the presence of dengue viral RNA by RT-PCR. Such RNA was found in 13 of 18 spleen specimens, 7 of 16 mesenteric lymph node specimens, and 14 of 18 liver specimens (232). The unusual increase of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in DHF patients is evidence of abnormal liver function (82, 112, 144, 173, 212). Hepatomegaly and liver dysfunction have a higher incidence in DHF patients than in dengue fever patients, suggesting that liver dysfunction may be related to DHF severity (52, 112, 118, 173, 185, 186, 190, 231, 260). Investigations of liver histopathology in cases of dengue virus infection revealed liver injury and dengue viral antigen in hepatocytes (11, 67, 112, 115, 144, 215). Additional studies directly suggest that hepatocytes are the major sites of dengue virus replication in the liver, as these studies detected dengue viral antigen and RNA in hepatocytes (122, 198) and demonstrated induction of apoptosis upon dengue virus infection (52, 185-187). Antigens in the blood do not come into direct contact with

hepatocytes, however. Liver sinusoidal endothelial cells (LSECs) form a barrier between hepatocytes and antigens in the blood.

Despite the liver being reported as a target organ of dengue virus infection, especially in severe and fatal cases, little information exists on the interaction between dengue virus and liver cells, especially in regards to the nature of the moieties facilitating the entry of the virus. Heparan sulfate, the ubiquitous glycosminoglycan which serves as a host cell binding site for numerous pathogenic agents, appears to be involved in dengue virus entry into liver cells (41), although the contribution of heparan sulfate to internalization of dengue virus appears to vary in a serotype-specific manner (171, 260). This precise serotype specificity is believed to be due to the high variation in amino acid sequences around the direct host cell binding domain among dengue serotype envelope proteins (54). Such variation might lead to the serotype specificity in host cell receptor recognition.

In addition to heparan sulfate, another possible receptor that dengue virus may be exploiting to infect liver cells is L-SIGN (Liver/lymph node specific ICAM-3 grabbing non-integrin). Unlike heparan sulfate, no serotype specificity has been shown with L-SIGN, as all four dengue serotypes can use L-SIGN to mediate infection (210, 257). L-SIGN is a calcium dependent C-type lectin expressed on endothelial cells of the lymph nodes and liver and shares 77% amino acid sequence identity with DC-SIGN. Within the liver, L-SIGN is expressed by liver sinusoidal endothelial cells (LSECs). These cells are specialized endothelial cells located in hepatic sinusoids that process blood-borne antigens found in circulation. Liver sinusoids are lined by LSECs forming an endothelial barrier that separates the sinusoid lumen and hepatic blood flow from hepatocytes. LSECs provide a diffusional barrier to viruses but also mediate transcytosis in a receptor-

driven process (135). Studies with hepatotropic viruses hepatitis B and C demonstrate that L-SIGN captures the viruses and, via receptor-mediated trancytosis, efficiently transinfects adjacent hepatocytes (29, 51, 77, 134). The ability L-SIGN to chaperone endocytosed virus past the lysosomal pathway may be central to its function as a capture receptor for dengue. This could explain how dengue virus gains access and infects hepatocytes. Unlike L-SIGN, model ligands are routed to late endosomes/lysosomes for degradation after DC-SIGN binding (66); however, DC-SIGN-bound HIV, for example, retains infectivity for prolonged periods (78). It is important to determine the molecular events that ensue after dengue binding to L-SIGN and DC-SIGN, as these events could impact dengue pathogenesis.

LSECs share a variety of unique features with dendritic cells, including scavenger function and antigen-presentation to $CD4^+$ and $CD8^+$ T cells (167). LSECs serve as potent antigen-presenting cells, as they constitutively express Class II molecules on their surface. Unlike dendritic cells, however, LSECs modulate T-cell responses to blood-borne antigens via induction of T cell tolerance(134-136, 167, 168). Naïve $CD4^+$ T cells primed by LSECs fail to develop into effector Th1 cells, and instead show a Th0 phenotype characterized by the expression of IL-10, IL-4, and IFNγ upon restimulation. Naïve $CD8^+$ T cells primed by LSECs lose the ability to express effector cytokines such as IFNγ and IL-2 following antigen-specific restimulation and also lose their cytotoxic activity against specific target cells. Thus, LSECs contribute to antigen-specific T cell-mediated immune tolerance to blood-borne antigens.

Taken together, infection of LSECs by dengue virus could result in the infection of hepatocytes and subsequent liver damage seen in severe disease and demonstrated by autopsy studies, histopathological findings, and clinical case reports (31, 50, 80, 96, 116,

154, 179, 202, 209, 217, 218, 233, 251, 254, 271, 272, 275). Infection of LSECs may also result in the induction of immune tolerance to the infecting dengue virus contributing to the increased viremia seen in severe disease (269). In fact, recently published data demonstrate that, in fact, low-passage dengue virus does efficiently replicate in LSECs (218). It will be necessary to follow up those results with studies that examine the effects of dengue infection of LSECs to determine the net result on immune function, but the literature supports LSEC induction of immune tolerance (134-136, 167).

Prior studies of dengue viruses have suggested that genetic differences among strains can be associated with attenuation, virulence, and epidemic potential (36, 42, 177, 182, 219, 230). Genetic analysis has revealed consistent differences in envelope glycoprotein between dengue 2 strains associated with DHF epidemics (Asian genotype) and dengue 2 strains only associated with DF (American genotype). American genotypes of dengue 2 virus consistently have the following amino acids – E81-Thr, E139-Val, E162-Val, and E390-Asp, whereas at those same residues Asian genotypes have E81-Ser, E139-Iso, E162-Iso, and E390-Asn. Our results show that the Asian genotype had higher infection rates for both DC-SIGN and L-SIGN bearing cells than the American genotype, when compared on the basis of equivalent molecular inocula. However, the Asian genotype, presumed to be more virulent given its association with DHF epidemics, appeared to make preferential use of L-SIGN compared with DC-SIGN as an attachment receptor whereas the American genotype exhibited the reverse infection phenotype.

The observed amino acid difference between Asian and American viruses at position E390 has been considered to be of major importance as a determinant of DHF in cases of secondary DEN2 infection (158, 240), but never proven. Residue E390 is located in the C-terminal domain III, which contains residues implicated as determinants

for host range, tropism, and virulence (224). For other dengue serotypes this pivotal residue is located within a few amino acids of 390. For dengue 3, it is at position E389 and the amino acid located there varies between strains also (201). Unfortunately, to date, most of the viral strain pathogenesis research has focused on serotype 2 American and Asian strains and little is known about differing virulence among strains of serotypes 1, 3 and 4. Results presented in this dissertation demonstrate that Tonga 74, a representative American genotype dengue 2 virus, showed the same infection phenotype as wild-type American IQT virus. However, when the amino acid at E390 was changed from negatively charged American aspartic acid to the neutral Asian asparagine, the *in vitro* infection phenotype changed from preferential DC-SIGN infection to preferential L-SIGN infection, equivalent to that demonstrated by the native Asian virus. Thus, the results shown in this dissertation demonstrate that the single mutation in E390 to Asian asparagine confers the Asian *in vitro* infection phenotype with regard to SIGN lectin interaction.

In a human infection, this preference of Asian virus for cells bearing the L-SIGN putative attachment receptor could contribute to the infection of hepatocytes via LSECs and subsequent liver damage that ensues in severe disease and has been demonstrated by autopsy studies, histopathological findings, and clinical case reports (31, 50, 80, 96, 116, 154, 179, 202, 209, 217, 218, 233, 251, 254, 271, 272, 275). While American viruses can also utilize L-SIGN, our results show that their efficiency of L-SIGN utilization is much lower than Asian viruses. Asian viruses would have significantly greater infection levels of LSECs, and could contribute to the increased viremia seen in severe dengue disease via two potential mechanisms: (1) increased viral replication due to increased infection efficiency in a larger cellular compartment compared with strains preferentially using

DC-SIGN, expression of which is restricted to dendritic cells, and (2) subsequently dampened immune response to novel epitopes specific to the virus causing the secondary infection, mediated by LSEC-induced tolerization of naïve T cells. Thus, preferential utilization of L-SIGN by the Asian genotype could be a determinant of disease pathogenesis.

Given the specificity with which carbohydrates interact with lectins, different lectin preferences of Asian and American genotype dengue 2 viruses may be due to differential glycosylation. Altmeyer and colleagues analyzed the capacity of DC-SIGN to bind and facilitate infection of dendritic cells by dengue virus grown in insect cells (134-136, 167). In insect cells, the E protein acquires one (Asn-67) or two (Asn-67 and Asn-153) trimannosylated N-glycans depending on the strain. When dengue viral strains bear two N-glycans, DC-SIGN receptor is used more efficiently suggesting that multiple contacts between E protein N-glycans and the oligomeric carbohydrate recognition domain of DC-SIGN are required.

Determining whether Asian and American strains are differentially glycosylated merits further study. It is certainly plausible that dengue viral glycoprotein N-glycan composition could vary between isolates, and these differences could impact lectin-binding specificity either directly or indirectly such that either L-SIGN or DC-SIGN could be preferentially targeted. The change from acidic Asp (American) to neutral Asn (Asian) in E390 could directly affect binding to lectins because changes in charge can affect conformation and E390 is spatially located next to the glycosylation region on the dimer protein, and also the glycosylation region on a neighboring dimer (200, 201, 224, 288, 289). Indirectly, the change in charge between American and Asian dengue 2 at

E390 may affect conformation and as a result, affect how Asn-67 and or Asn-153 are glycosylated.

Figure 4-7 outlines the glycosylation process that would be involved in attaching the oligosaccharide chain to dengue residues Asn-67 and Asn-153. A long series of modifications of the polysaccharide chain occurs in both the endoplasmic reticulum and Golgi apparatus. Each step is carried out by a different enzyme that is particular to the cell type in which replication occurs. Sugar processing can be affected by steric hindrance, such that in certain conditions, an enzyme may not gain access to the sugars that need to be added to or cleaved. Thus, it is plausible that a change in charge at E390 could affect protein conformation, which would result in differential glycosylation and consequently differential lectin tropism.

In addition to lectin tropism, differential glycosylation could also affect neutralization as the binding domain (domain III) of the envelope glycoprotein is not universally distributed and the domains are in a different orientation. Some areas form what is referred to as a 5-fold axis, which becomes crowded with the attached oligosaccharides. Such crowding could block antibodies from accessing their epitope, depending on the number and type of sugar moieties attached to the glycoprotein, and thus antibody binding due to steric hindrance could ensue (60, 142, 229). For steric hindrance to occur, the antibody-binding site need not be located in close proximity to the glycosylation region. The antibody-binding region could be located near the glycosylation site on the protein it is dimered with, or a neighboring dimer (Figure 4-8). Our results show differential neutralization of Asian and American viruses using mAbs 3H5 and 4G2. Dengue 2-specific mAb 3H5 binds to an epitope located around residue E390. When the native confirmation of the dengue 2 envelope glycoprotein is examined,

residue E390 is located in close proximity to both Asn-67 and Asn-153 glycosylation sites (Figure 4-9). Flavivirus group reactive mAb 4G2 binds to an epitope located in the highly conserved fusion peptide region of Domain II (230) (Figure 4-10). In the native dimer structure, the fusion peptide loop is packed against the other subunit at DI residue Asn-153 (53). Thus, various dengue virus strains with potentially different glycosylation patterns could impact neutralization results

Epidemiological studies have shown that pre-existing immunity to dengue virus is a major risk factor for dengue hemorrhagic fever. Thus, having a previous dengue infection increases the risk of severe disease upon subsequent infection with a heterologous serotype. Two immunopathogenic theories were borne from these epidemiological observations – antibody dependent enhancement and T-cell mediated pathogenesis. Contrary to the immunopathology theories however, dengue hemorrhagic fever does not always occur in areas where multiple serotypes of dengue virus co-circulate. Genetic analysis has revealed that the strain of dengue 2 associated with dengue hemorrhagic fever epidemics belongs to the Asian genotype, while the American genotype has never resulted in severe disease. In the context of secondary dengue 2 infections, our differential tropism and neutralization results could possibly bridge these two main pathogenesis theories. Genetic differences between American and Asian dengue 2 genotype viruses may result in differential utilization efficiencies of DC-SIGN and L-SIGN and differential neutralization profiles that correlate with disease severity. Asian dengue 2 viruses associated with more severe dengue disease appear to preferentially utilize L-SIGN as a receptor mediating dengue virus infection. Thus, infection of LSECs and subsequent immune tolerance, and infection of hepatocytes via

trancytosis and consequent liver damage, would be seen more in Asian infections and may explain the increased disease severity and pathogenicity.

It is important to remember that the immune response is a complex system of check and balances – it is never all or nothing. For every cell and every cytokine that upregulates the immune response, there is another cell type and another cytokine that down-regulates the response. Most of the time a dengue infection is cleared without causing undue harm of the host. Sometimes however, something goes wrong and people die of a dengue infection. This occurs, on average, in about 5% of dengue cases (282). Figure 4-11 illustrates the potential mechanisms of secondary dengue 2 pathogenesis, albeit overly simplified, and how some of the immune components may interact and play a role in dengue disease. Based on past knowledge and research using dendritic cells, dengue infection of dendritic cells via the DC-SIGN would most likely result in the induction of an immune response. Viruses such as the Asian strains of dengue 2 that can utilize both DC-SIGN and L-SIGN as efficient receptors to mediate infection would result in an expansion of replication sites and ultimately increased viremia. Additionally, infection of LSECs via L-SIGN may lead to T-cell tolerance to the infecting virus, which would temporarily dampen the immune response to the invading virus while cross-reactive memory T-cells could be stimulated, resulting in a relatively ineffective initial immune response to the infecting virus while maintaining a robust cytokine response – cytokines which have been shown to be potential mediators of the plasma leakage observed in severe dengue disease.

Pre-existing heterologous antibodies, under conditions of antibody specificity or concentration where neutralization does not occur, may facilitate viral entry of FcR-bearing cells (ADE theory), resulting in an expansion of replication sites and a cytokine

response that could mediate plasma leakage. Furthermore, antibody bound to virus also increases viral uptake by LSECs via Fcg-R, enhancing dengue infection of LSECs. Moreover, even if the antibodies had the potential to neutralize the heterologous dengue virus, the affinity of the virus for L-SIGN may be greater than the affinity the antibody has for its epitope on the virus, which would statistically result in reduced neutralization and, instead, infection of LSECs. The end result of all of these possibilities would lead to increased viremia, which has been shown to be associated with more severe disease (269). Of course there are host factors and genetic susceptibilities to consider as well, however the role those factors may play in dengue pathogenesis is beyond the scope of this dissertation.

Despite the fact that one could consider the differences we found in lectin tropism between Asian and American strains as relatively subtle, the differences were statistically significant and biologically reasonable. Only a small percentage, around 5%, of dengue fever cases progress to the more severe form of disease. The immune tolerance induced by LSECs could impact a localized immune response sufficiently to temporarily dampen the response to the infecting dengue virus. The intravenous injection of antigen has been shown to be a potent means of inducing T cell suppression (117, 160) and, in the case of intravenous injection of ovalbumin, after only one hour the majority of the antigen was found in LSECs (140). LSECs transfer hepatitis B to adjacent hepatocytes and chronic infection is associated with a systemic lack of T-cell mediated immunity. Thus, LSECs appear capable of having a powerful influence on the immune response, and in conjunction with the relative efficiency of Asian dengue 2 virus for L-SIGN, this preference could sufficiently to tip the scales for an unfortunate few percent of cases to develop severe disease.

A relevant question following any *in vitro* research is whether the *in vitro* results have any biological relevance to what occurs *in vivo*. Until large-scale population-based studies are conducted, we have no way of knowing that with any certainty. However, a recently published article in Nature Genetics found an association between host genetics and disease severity that compliments our proposed mechanism of secondary dengue pathogenesis (Figure 4-11) and lends confidence that our work could indeed be relevant to *in vivo* human infection (239). In this study, a variant in the DC-SIGN (CD209) promoter was found to be associated with dengue disease severity. A G allele was associated with strong protection against dengue fever (OR-4.90), yet ironically was also associated with an increased risk for dengue hemorrhagic fever (OR=5.84). Having a G allele resulted in decreased expression of CD209 and therefore, lower susceptibility of dendritic cells to dengue virus. However, that begged the question as to why and how the G allele could be associated with an increased risk of DHF. The authors proposed that their results could be due to different viral strains or the virus could be using an alternate viral entry pathway. In light of our data, we would suggest that the explanation includes both conditions – both the strain of the virus and an alternate viral entry pathway. Given the reduced dendritic cell availability, an American dengue 2 virus would have a difficult time securing and sustaining an infection, however, an Asian dengue 2 virus would almost exclusively utilize L-SIGN, whereby the likelihood of severe disease is further increased.

Although this work represents an important contribution to the field of dengue research, much more work remains in order to define the specific contributions of differential tropism and neutralization sensitivity to dengue pathogenesis. While the DC-SIGN- and L-SIGN-transfected Raji cells used in these studies are suitable surrogates to

examine dengue tropism and neutralization, it would be advantageous to use primary dendritic and liver sinusoidal endothelial cell cultures for future research. Infection and replication in primary human LSECs must be examined and the role that these cells may play in the dengue immune response, such as initiating T-cell tolerance and the mechanism by which it occurs, must be further elucidated.

It would be beneficial to determine the relative amounts of defective particles in the virus preparations to determine whether the reduction in infectivity of the American IQT virus is due to a larger ratio of defective to infective particles compared to the Asian OBS virus. Since little is known about the efficiency of the DC-SIGN and L-SIGN lectin receptors, characterizing the viruses in terms of defective particles would be beneficial to rule out the possibility that the results could be due to differential interaction between defective and infective particles and the lectin receptors. The virus output from infected LSECs also needs to be examined and quantitated in order to show that replication in these cell types would in fact contribute to increased viremia due to an expansion of replication sites. In addition, given that the early illness serum was acquired in a dengue hyperendemic region, the sera could be further examined for the presence of neutralizing activity to other dengue serotypes or even tetravalent immunity, which could illuminate additional information about the dengue immune response and disease severity. Obtaining immediate pre-infection serum would also be beneficial, as this serum would clearly not contain any antibodies to the current infection and would be completely devoid of virus. However, pre-infection serum is acquired under very limited circumstances and requires very large prospective studies.

Furthermore, this thesis was limited to genotypes within the dengue 2 serotype given that there exists a clear association of severe disease with the Asian genotype and

only mild disease associated with the American genotype. However, other dengue serotypes should be studied to determine potential lectin preference and neutralization sensitivity among the more virulent strains of dengue 3 for example. Viral strains' growth in different cell lines, such as mammalian versus mosquito cells could also be examined for effects on tropism and neutralization, as presumably, viral envelope glycosylation patterns will be different. Comparing the oligosaccharides and carbohydrate moieties for the different dengue strains would also be beneficial and would further elucidate the effects of glycosylation. Other C-type lectins could be examined as well, including the recently discovered LSECtin, which has the closest homology to L-SIGN.

This is a very exciting time to be conducting dengue research, as much is being discovered about the virus, the host, and the transmission cycle. There is also a pressing need to develop vaccines to protect the millions of adults and children from infection and death. Central to the development of vaccines is the induction of neutralizing antibodies as opposed to enhancing antibodies. The phenomenon of antibody-dependent enhancement has greatly slowed the field-testing of vaccine candidates. Much work needs to be done to thoroughly elucidate the conditions in which ADE occurs and the means of avoiding its induction in the formulation of vaccine candidates. Hopefully the work presented in this dissertation will help contribute to those future efforts.

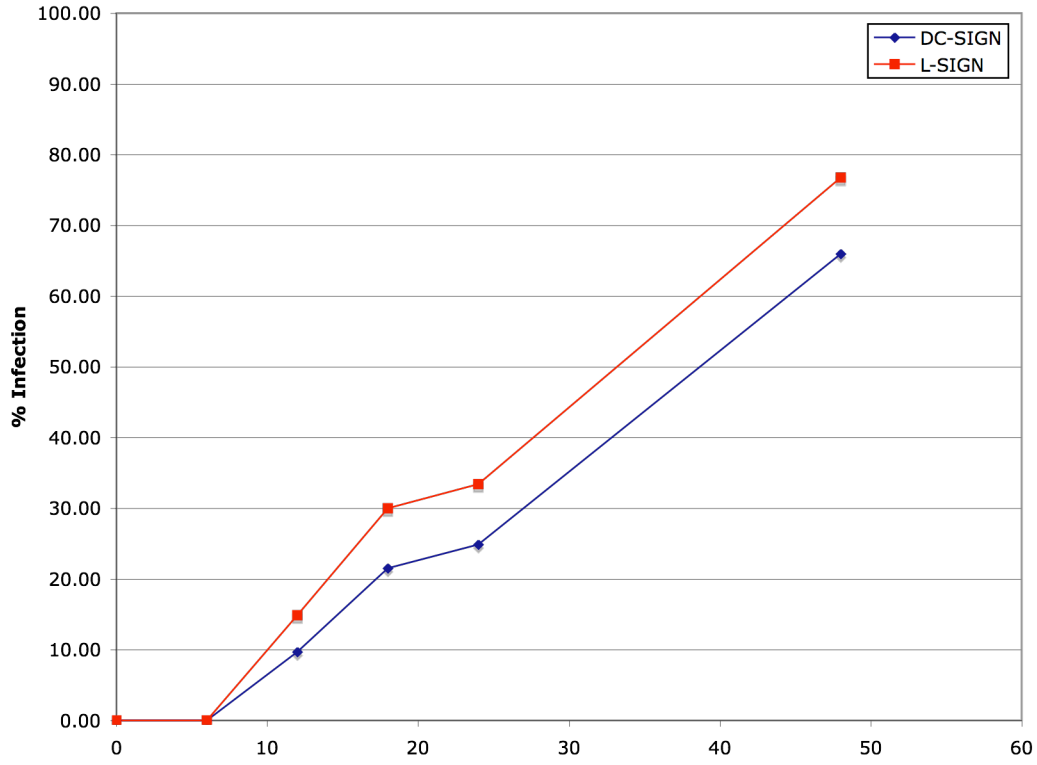


Figure 4-1: DEN2 S16803 at MOI=0.08 was added to DC-SIGN or L-SIGN transfected Raji cells and harvested after 0, 6, 12, 18, 24 or 48 hours. Multiple rounds of replication occur after 24 hours.

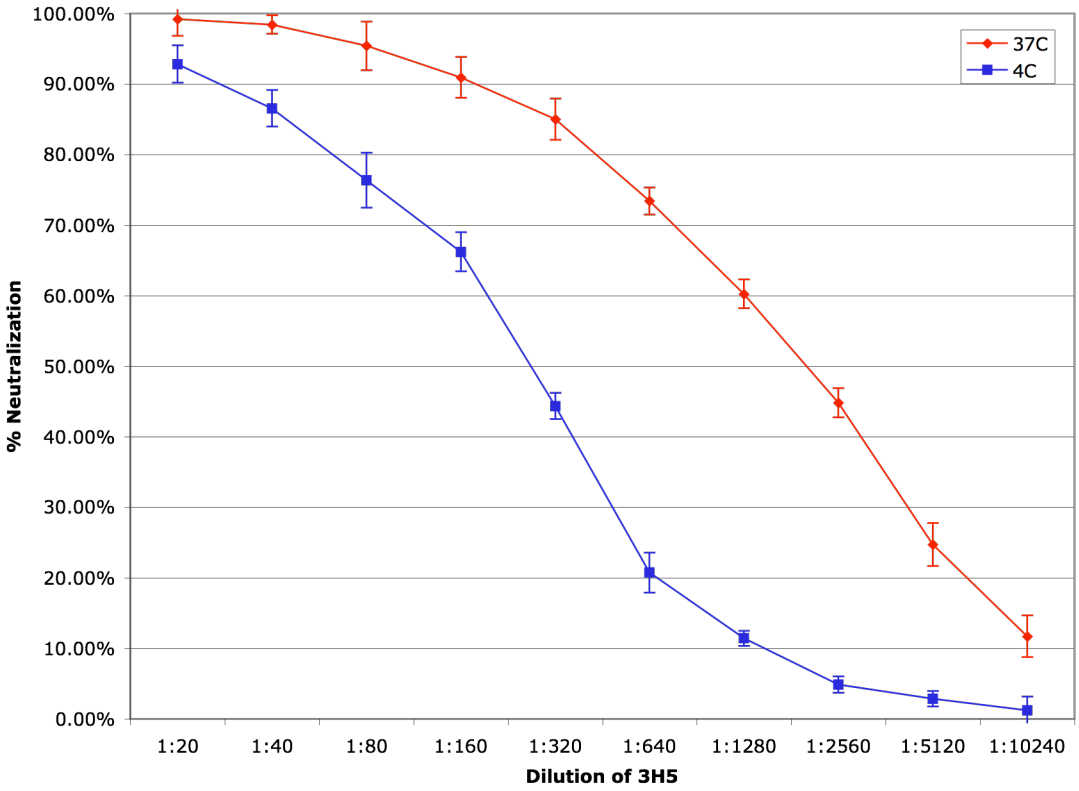


Figure 4-2: Incubation Temperature Optimization. DEN2 S16803 at MOI=0.08 was incubated with neutralizing 3H5 at either 37C or 4C. DC-SIGN transfected Raji cells were added after 30 minutes and the mixture was harvested for intracellular preM-Ag staining after 20 hours. As neutralization kinetics would dictate, more neutralization occurred at 37C than 4C. The 37C incubation temperature was chosen for future experiments, as this temperature increased the neutralization assay's sensitivity.

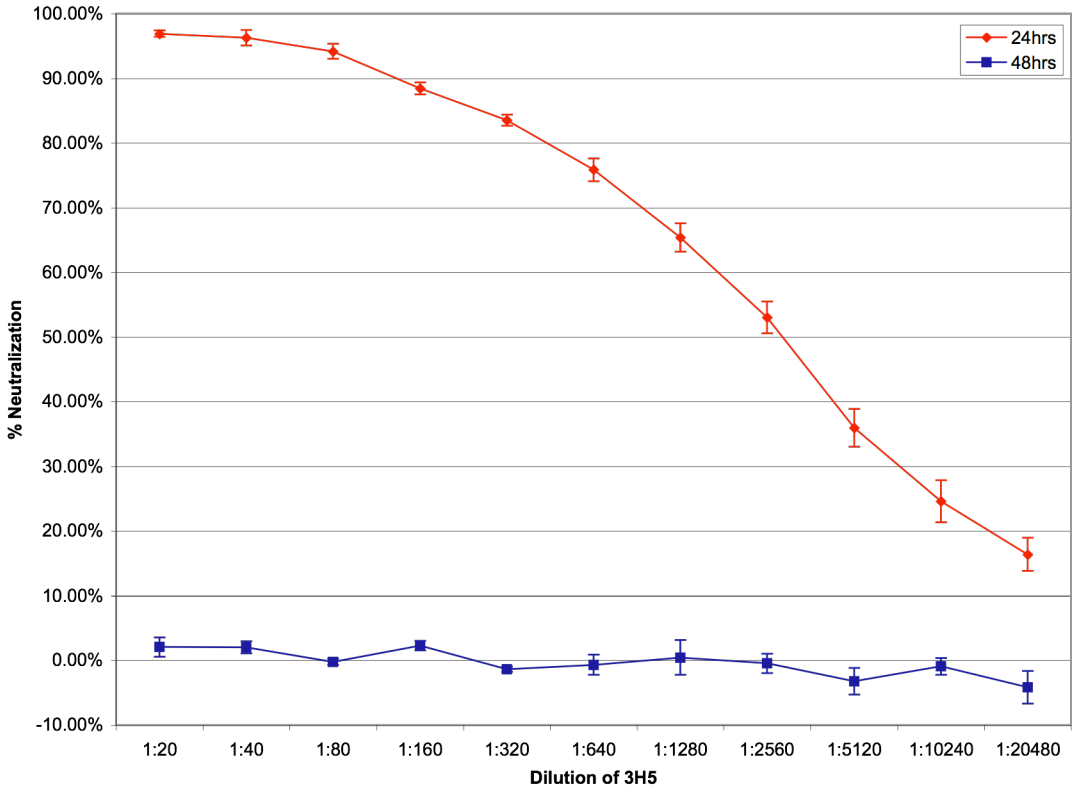


Figure 4-3: Incubation Time Optimization. DEN2 S16803 at MOI=0.08 was incubated with neutralizing 3H5 at either 37C. DC-SIGN transfected Raji cells were added after 30 minutes and the mixture was harvested for intracellular preM-Ag staining after either 24 or 48 hours. After 48 hours, neutralization results were masked by multiple rounds of replication.

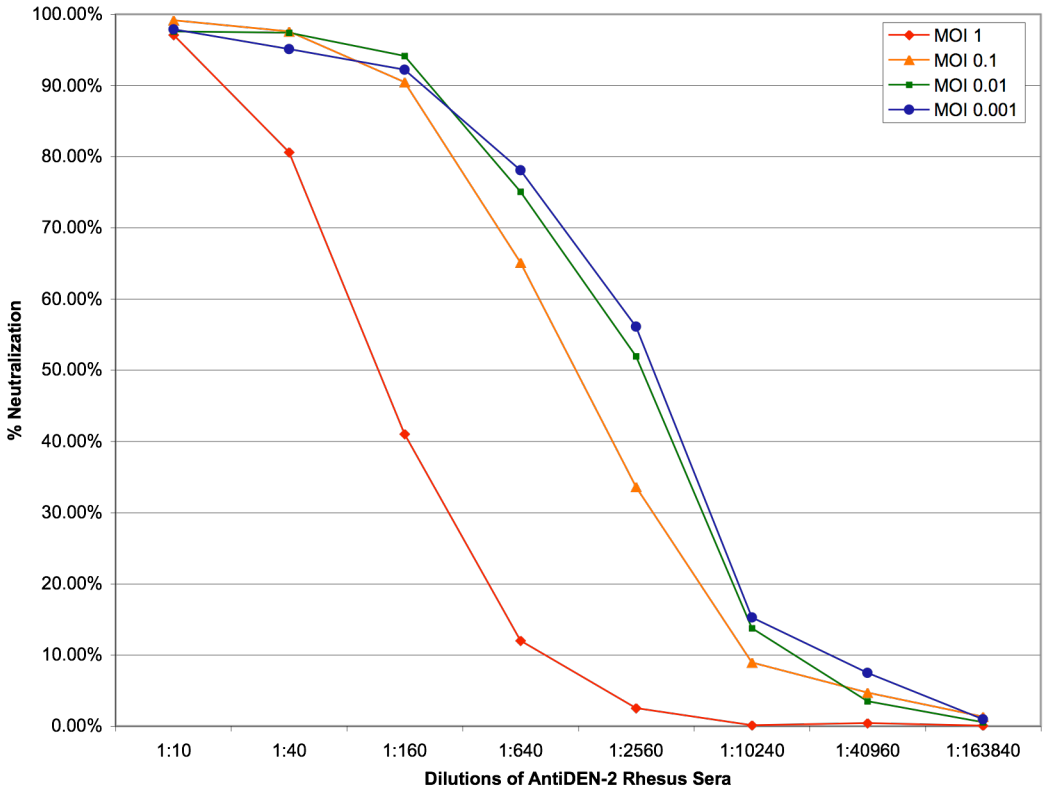


Figure 4-4: Varying MOIs of DEN2 S16803 were exposed to serial dilutions of antiDEN-2 rhesus sera. As the MOI, or number of plaque forming units, decreases, the neutralization curve shifts to the right, presumably because the amount of antibodies become in excess and saturates the amount of virus. Once the antibodies are in excess, the neutralization results become less sensitive to changes in MOI. The MOI at which this occurs is the MOI that results in a linear range of viral infection.

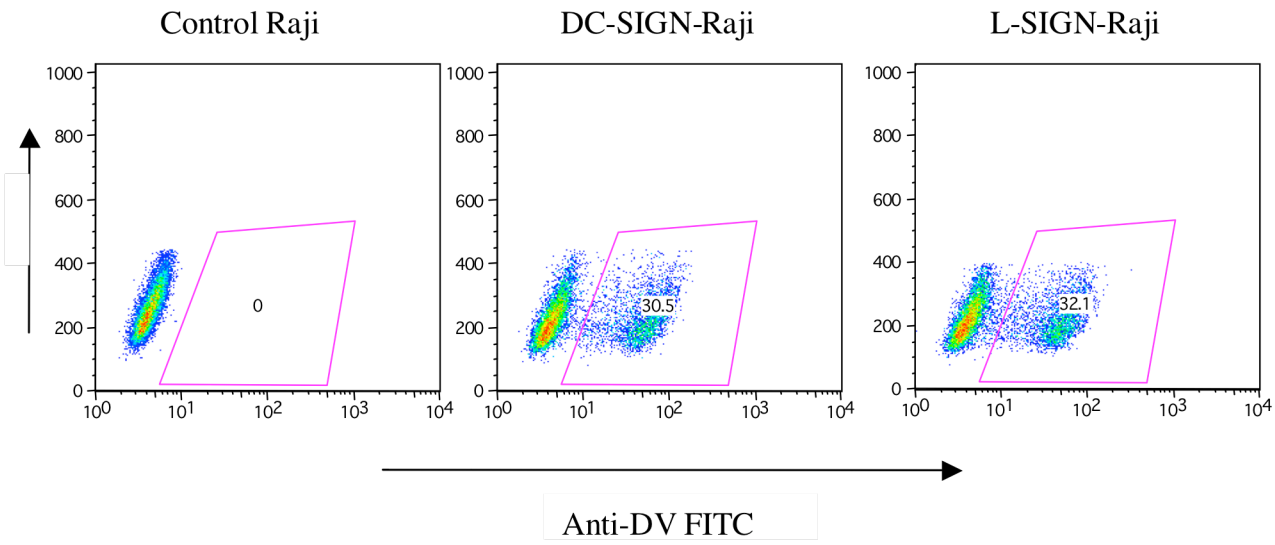


Figure 4-5: SIGN-transfected and control Raji cell lines were exposed to DEN2 S16803 at MOI=0.08 or mock infected. Scatterplots show the cell lines gated on viability and 2H2-FITC expression (anti-preM-Ag) (A) The Raji cell line, normally resistant to infection, becomes permissive upon DC-SIGN or L-SIGN transfection.

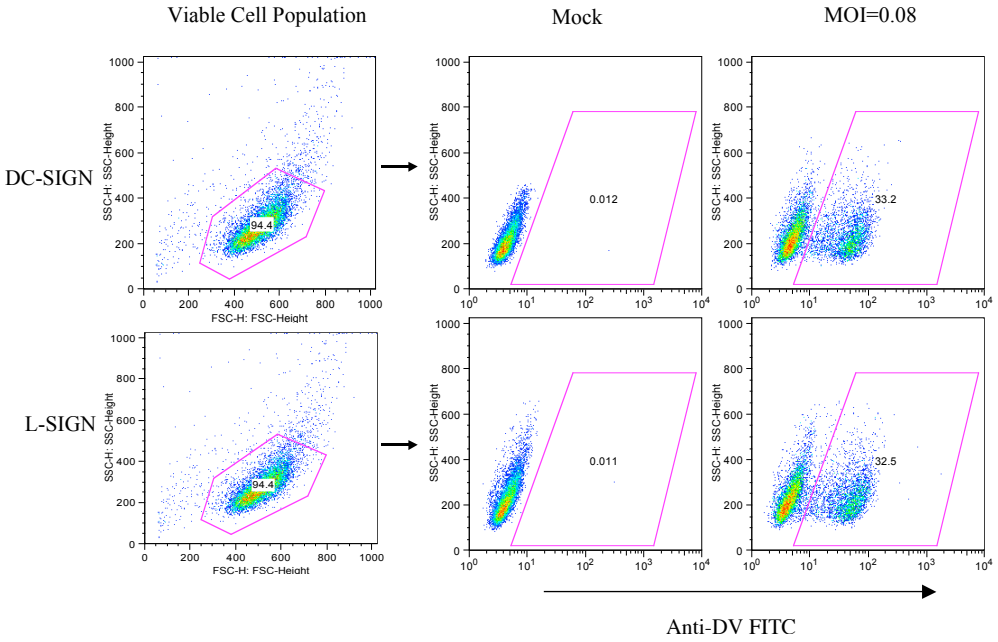


Figure 4-6: SIGN-transfected and control Raji cell lines were exposed to DEN2 S16803 at MOI=0.08 or mock infected. Scatterplots show the cell lines gated on viability and 2H2-FITC expression (anti-preM-Ag). Clear discrimination of preM-Ag-positive cells from background is demonstrated.

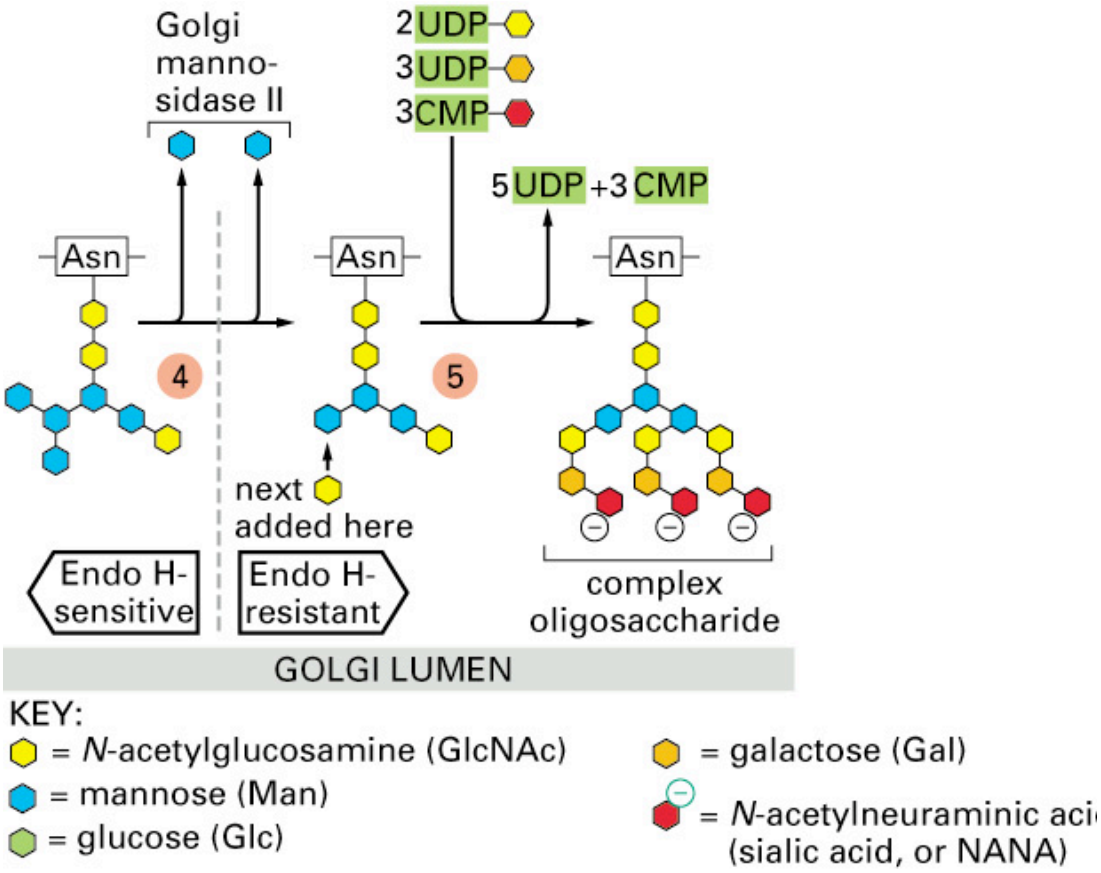


Figure 4-7: N-linked glycosylation process. The oligosaccharide chain is attached to the asparagine within the context of the sequence Asn-X-Ser/Thr within the dengue envelope protein while being processed in the endoplasmic reticulum. A long series of modifications of the polysaccharide chain occurs in the endoplasmic reticulum and the Golgi apparatus. Each step is carried out by a different enzyme specific to the cell type in which the process occurs. (Molecular Biology of the Cell, 4th Edition, Figure 13-26 part 2).

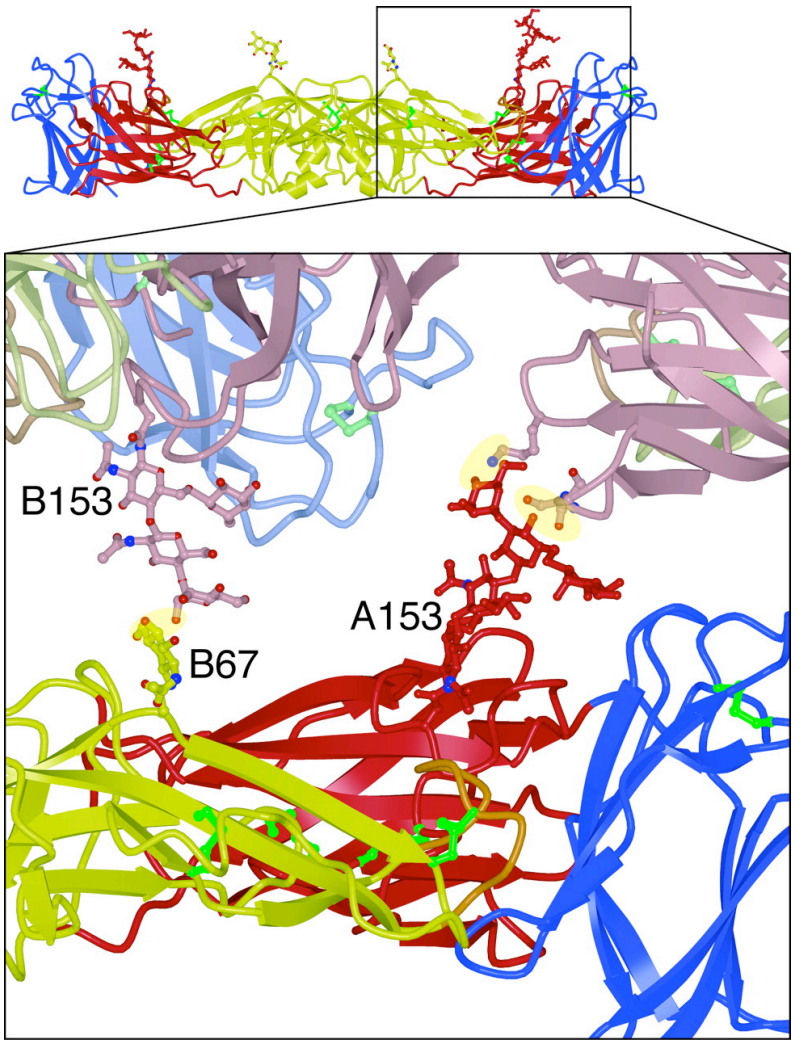


Figure 4-8: A close-up of the E dimer (in red, yellow, and blue for domains I, II, and III, respectively) shows that both glycans form crystal contacts with an adjacent molecule in the crystal (shown in lighter colors). Mannose residues in the glycan on Asn-153 of chain A form hydrogen bonds with the main chain and side-chain oxygen atoms of Ser-16 and the side-chain amine of Lys-36. The N-acetylglucosamine linked to Asn-67 of chain B forms a hydrogen bond with a mannose in the glycan on Asn-153 of chain B of a neighboring molecule in the crystal. (Modis, Y. et. al. 2005. Variable surface epitopes in the crystal structure of dengue virus type 3-envelope glycoprotein. J Virol, 79 (2): 1223-31).

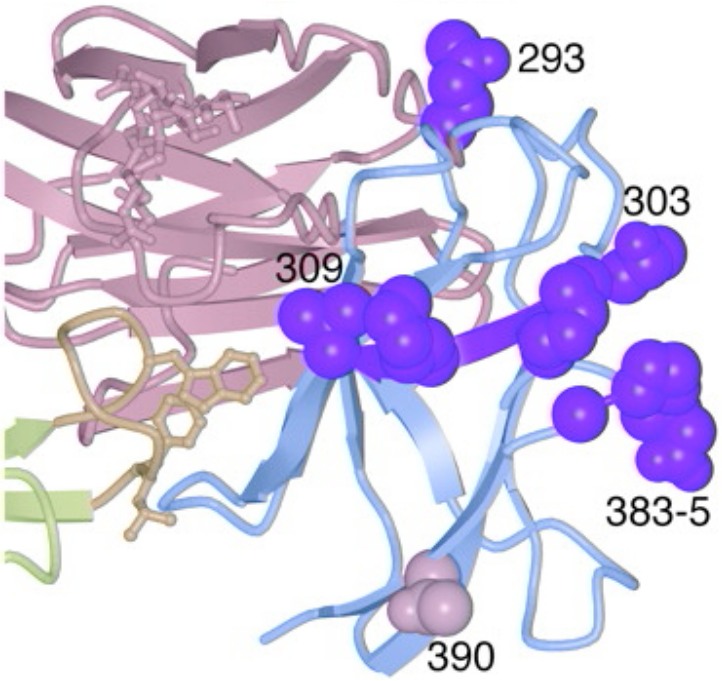


Figure 4-9: Stereoscopic view and close-up of DEN-2 domain III 3H5 neutralization-escape mutants. Three serotype-specific epitopes have been reported: residue 293, residues 303 to 309, and residues 383 to 385. Side chains in all three epitopes are shown on the DEN-2 E structure in magenta in space-filling representation. Changes at residue 390 (in pink), also unconserved and exposed, correlate with changes in virulence. Oligosaccharides on Asn-67 and Asn-153 are spatially located close to the proposed 3H5-binding site. (Modis, Y. et.al. 2005. Variable surface epitopes in the crystal structure of dengue virus type 3-envelope glycoprotein. J Virol 79 (2): 1223-31).

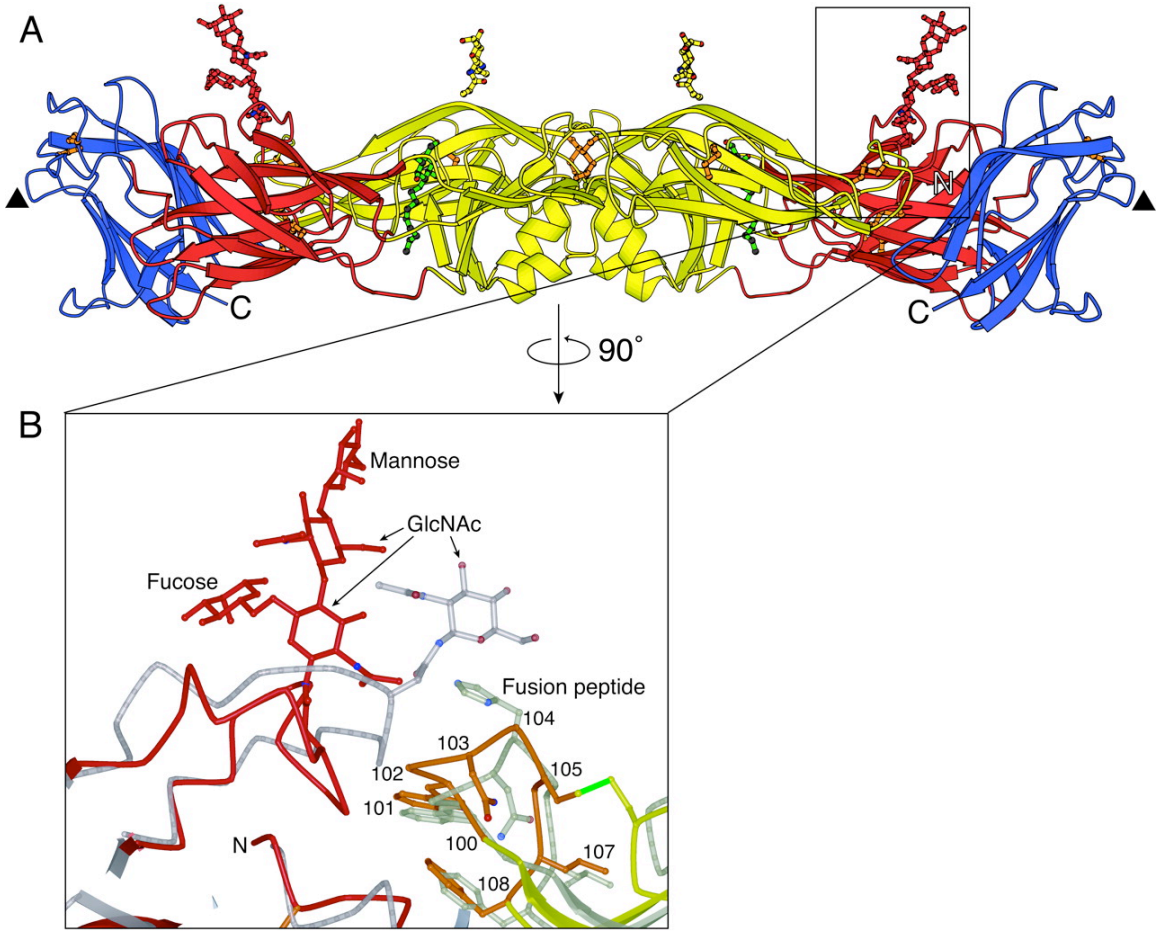


Figure 4-10: The glycan at residue Asn-153 in DEN 2 E protein. (A) Both glycans are perpendicular to the viral surface. Domain is red, domain II is yellow, and domain III is blue. Disulfide bridges are orange. The hydrophobic pocket underneath the hairpin is green. A putative receptor-binding loop in domain III (residues 382–385) is marked with a triangle. (B) Enlargement of the area surrounding the glycan at residue 153, with the structure of TBE E protein superimposed (gray) onto domain I of DEN E. The fusion peptide is highlighted in orange. The disulfide bridge between residues 92 and 105 is green. (Modis, Y. et. al., 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. PNAS 100 (12): 6986-6991).

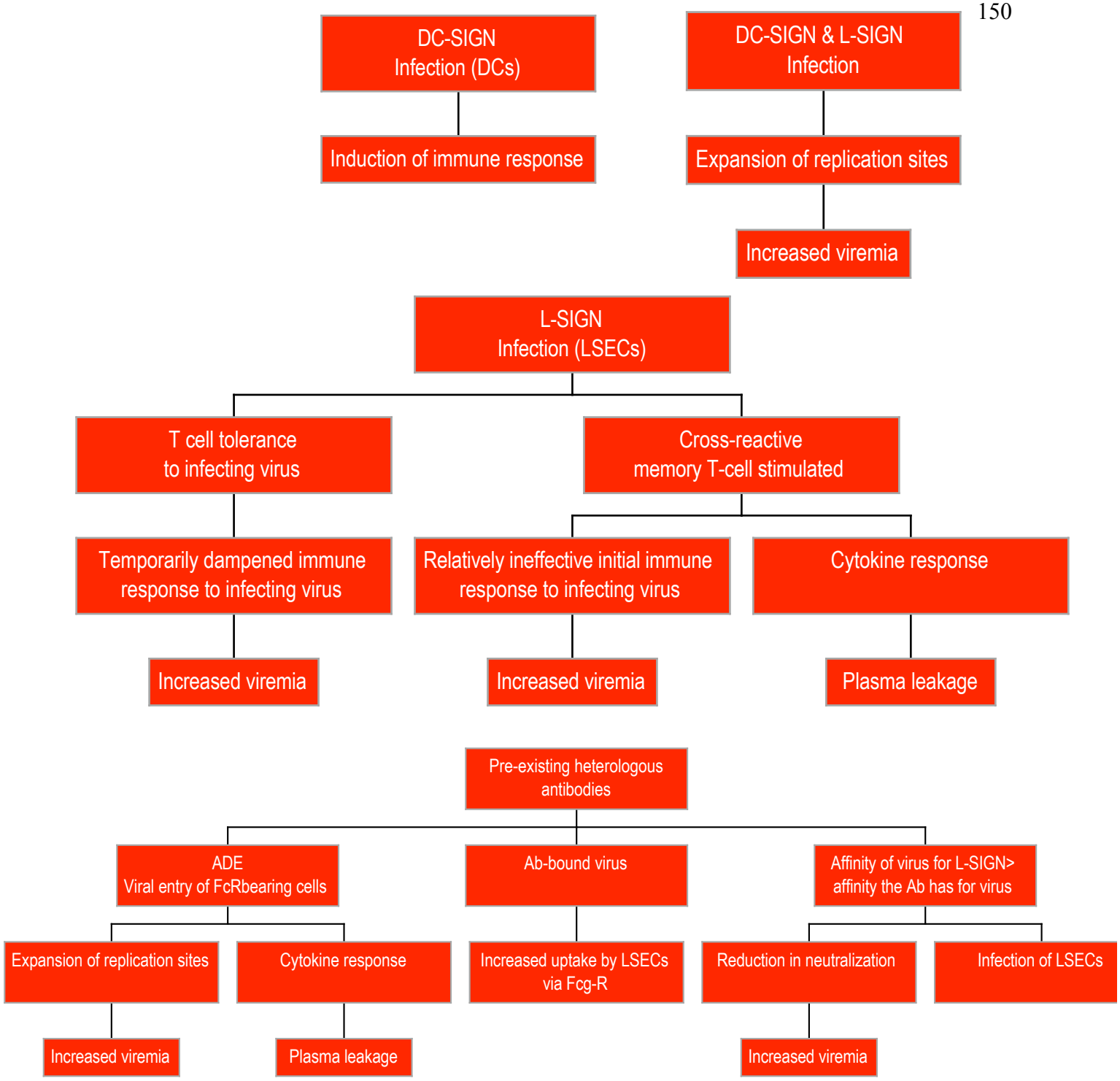


Figure 4-11: Potential Mechanism of Secondary Dengue 2 Disease Pathogenesis.

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